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(54) Title: METHODS FOR ENGINEERING ANTIGEN-PRESENTING CELLS

(57) Abstract

A protein transfer method for producing a cell having a defined MHC: nominal antigen peptide or costimulator on its membrane.



phβ2M/Pac



pHLA-A2.1:DAF-S/Pac



pHLA-A2.1/Pac



pHph/Pac
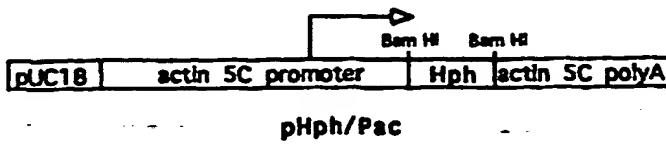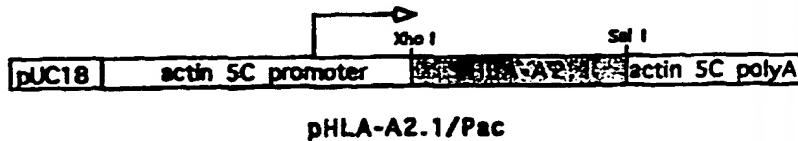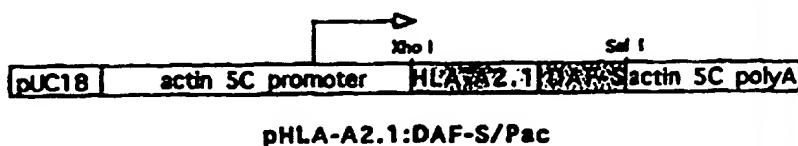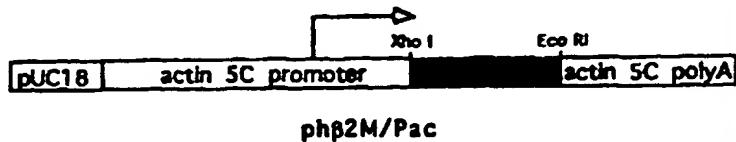
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## DESCRIPTION

## Methods For Engineering Antigen-Presenting Cells

### Field of the Invention

This invention relates to methods and reagents for engineering antigen-presenting cells ("APCs") for purposes of immunotherapy.

### 5 Background of the Invention

The following is a general discussion of relevant art, none of which is admitted to be prior art to the invention.

An objective in the field of immunotherapy is the

10 development of strategies for activating or inhibiting T-cells that are therapeutic or pathogenic, respectively. APCs provide one efficient means for accessing antigen-specific T-cells.

In general, APCs are responsible for initiating most

15 immune responses through their pivotal role in antigen presentation to T-cells. During antigen presentation, endogenously processed nominal antigen peptides associate intracellularly with either class I major histo-compatibility complex ("MHC") heterodimers or with class

20 II MHC heterodimers, and the resulting heterotrimeric complexes are then translocated to APC surfaces (reviewed in Germain and Margulies, 11 Annu. Rev. Immunol. 403, 1993; Germain 76 Cell 287, 1994). For a single immuno-genic protein that is pulsed with APCs, processing fre-

25 quently yields a diverse MHC:nominal antigen peptide repertoire, the composition of which may differ between different APCs (Falk et al., 348 Nature 248,1990; Falk et al., 174 J. Exp. Med. 425, 1991; Henderson et al., 255 Science 1264, 1992; Henderson et al., 90 Proc. Natl. Acad.

30 Sci. USA 10275, 1993; Hunt et al., 255 Science 1261, 1992; Rammensee et al., 11 Annu. Rev. Immunol. 213, 1993; Huczko et al., 151 J. Immunol. 2572, 1993).

In order to achieve a more defined MHC:nominal antigen
peptide repertoire on a given APC surface, it is
experimentally feasible to load exogenously synthesized
oligopeptides onto class I or class II MHC heterodimers
5   (Harding et al., 86 Proc. Natl. Acad. Sci. USA 4230,
1989). Such exogenously-loaded peptides do not require
intracellular proteolytic processing.

For purposes of antigen presentation, it is common to
employ one of two categories of cells, that is, cells that
10  naturally express MHC heterodimers and cells transfected
with an MHC gene expression cassette. In principle,
another category of an MHC-bearing cell is one in which
the MHC molecule has been exogenously attached to the cell
surface. A method for delivering an MHC molecule to an
15  APC surface has been described which entails the use of an
HLA-A2.1-streptavidin chemical conjugate which can be
added to a pre-biotinylated cell (Elliott and Eisen, 87
Proc. Natl. Acad. Sci. USA 5213, 1990). The chemical
moiety was shown to be alloantigenic, but no data was
20  presented that it can bind and present a nominal antigen
peptide.

Functional interactions between APCs and T-cells are
known to be mediated by both cell surface-associated and
soluble molecules. Antigen-specificity in this inter-
25  action is provided by the major histocompatibility com-
plex:nominal antigen peptide complex of the APC inter-
acting with the T-cell receptor (TCR) of the T-cell,
constituting a trimolecular axis. However, effective
interactions additionally require certain cell surface-
30  associated and soluble costimulator molecules of APCs
binding to receptors for these costimulators on T-cells.
Examples of known costimulators on APCs are B7 (Linsley et
al., 87 Proc. Natl. Acad. Sci. U.S.A. 5031, 1990); ICAM-I
(van Seventer et al., 144 J. Immunol. 4579, 1990); VCAM-I
35  (van Seventer et al., 174 J. Exp. Med. 901, 1991); LFA-3
(van Seventer et al., 21 Eur. J. Immunol. 1711, 1991;
fibronectin (Shimizu et al., 145 J. Immunol. 59, 1990;

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Nojima et al., 172 J. Exp. Med. 1185, 1990; Davis et al., 145 J. Immunol. 785, 1990).

An objective in the field of tumor immunotherapy is the development of strategies for enhancing tumor immuno-
5  genicity, with potential applications in both tumor prevention and cure.  In general, tumors that arise de novo are poorly immunogenic, thereby escaping host anti-tumor responses (Hewitt et al., 33 Br. J. Cancer 241, 1976).  The basis for this low immunogenicity is unclear.

10  Several experimental strategies have been described for enhancing tumor immunogenicity, for example, use of mutagen or drug treatment (Van Pel and Boon 79 Proc. Natl. Acad. Sci. USA 4718, 1982, Frost et al., 159 J. Exp. Med. 1491, 1984); by transfection with a foreign gene encoding

15  an exogenous antigen such as influenza hemagglutinin (Fearon et al., 38 Cancer Res. 2975, 1988); by reducing the expression of certain molecules in a tumor that regulate its differentiation state (Tykocinski & Ilan, PCT/US93/03637); by transferring a gene expressing a

20  lymphokine into a tumor, for example, interleukin-2 (Fearon et al., 60 Cell 397, 1990), interleukin-4 (Tepper et al., 57 Cell 503, 1989, Golumbek et al., 254 Science 713, 1991).

Summary of the Invention

25  Applicant has determined a method for delivering a nominal antigen peptide to a cell surface that is not contingent upon prior expression of MHC by these cells. Both the MHC polypeptide and the nominal antigen peptide are externally delivered to the cell surface; intracel-

30  lular protein processing pathways are not utilized.  The method is predicated upon the use of a membrane reincor-porable variant of an MHC polypeptide.  According to alternative embodiments of the present method, a nominal antigen peptide can be contacted with the MHC polypeptide

35  variant prior to, concurrent with, or subsequent to the membrane reincorporation event.  The MHC component is

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configured in a way that both facilitates its exogenous reincorporation into membranes and preserves its antigen presentation capacity. Hence, by such a strategy, APCs can in essence be "painted" with defined MHC:nominal
5   antigen peptide complexes. This method allows for the tailoring of the antigen repertoire of an APC and enhancement of its therapeutic efficacy.

Applicant has determined that certain MHC polypeptide derivative can be genetically engineered to be suitable
10  for use as a vehicle to deliver a nominal antigen peptide to a cell surface. Applicant believes that molecular topology substantially influences the MHC:nominal antigen peptide:T-cell receptor trimolecular axis and functional signaling through this axis. Hence, molecular topological
15  aspects of an MHC moiety are critical determinants of its capacity to present antigen to T-cells. This indicates that a subset of engineered MHC polypeptide derivatives will support efficient antigen presentation to T- cells. Such a subset is determined by routine screening of
20  different MHC classes using the techniques described below. The present invention discloses a class of MHC polypeptide derivatives which is particularly well-suited for effective antigen presentation. This class of MHC polypeptide derivatives has the added feature of reincor-
25  porability into cell membranes. This feature permits it to be coated, or "painted", onto the surface of a cell.

The present invention is based upon the finding that lipid-modified MHC heterodimers, which acquire the property of membrane reincorporability by virtue of their
30  lipid modification, retain an efficient antigen presentation function. A preferred lipid modification for this purpose is a glycosyl-phosphatidylinositol ("GPI") modification.

GPI-modified proteins are a class of native cell
35  surface molecules that can be exogenously reincorporated back into cell membranes after purification (Medof et al., 160 *J. Exp. Med.* 1558, 1984; Moran et al., 149 *J. Immunol.*

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1736, 1992; Zhang et al., 89 Proc. Natl. Acad. Sci. USA 5231, 1992; Bulow et al., 27 Biochemistry 2384, 1988; Hitsumoto et al., 5 Int. Immunol. 805, 1993). This property stems from their amphiphilic properties and their

5   solubility in exceedingly low detergent concentration, and as Applicant has found, in the complete absence of detergent. Protein transfer has been reported for a limited set of natural GPI-anchored proteins, including decay-accelerating factor ("DAF") (Medof et al., 160 J. Exp.

10  Med. 1558, 1984; Moran et al., 149 J. Immunol. 1736, 1992), Thy-1 (Zhang et al., 89 Proc. Natl. Acad. Sci. USA 5231, 1992), T. brucei variant surface glycoprotein (Bulow et al., 27 Biochemistry 2384, 1988), and mouse heat-stable antigen (Hitsumoto et al., 5 Int. Immunol. 805, 1993).

15  For DAF and heat stable antigen, biological functions have been demonstrated for the exogenously reincorporated proteins (Moran et al., 149 J. Immunol. 1736, 1992; Zhang et al., 89 Proc. Natl. Acad. Sci. USA 5231, 1992; Hitsumoto et al., 5 Int. Immunol. 805, 1993)).

20     Polypeptide sequences can be artificially GPI-modified by linking their coding sequences to a GPI modification signal sequence (Tykocinski et al., 85 Proc. Natl. Acad. Sci. USA 3555, 1988; Straus et al., 82 Proc. Natl. Acad. Sci. USA 6245, 1985; Caras et al., 238 Science

25  1280, 1987; Waneck et al., 85 Proc. Natl. Acad. Sci. USA 577, 1988). This finding has been substantiated by other more recent studies for a variety of target proteins (Lin et al., 249 Science 677, 1990; Sleckman et al., 147 J. Immunol. 428, 1991; Zhang et al., 115 J. Cell Biol. 75,

30  1991; Mann et al., 142 J. Immunol. 318, 1989; Matsui et al., 254 Science 1788, 1991; Wettstein et al., 174 J. Exp. Med. 219, 1991; Kemble et al., 122 J. Cell Biol. 1253, 1993; Weber et al., 210 Exp. Cell Res. 107, 1994; Huang et al., 31 Mol. Immunol. 1017, 1994; Scheirle et al., 149 J.

35  Immunol. 1994, 1992). Gene transfer of GPI-modified MHC is disclosed by Mann et al., 142 J. Immunol. 318, 1989; Matsui et al., 254 Science 1788, 1991; Wettstein et al.,

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174 <u>J. Exp. Med.</u> 219, 1991; Scheirle et al., 149 <u>J. Immunol.</u> 1994, 1992; and Huang et al., 31 <u>Mol. Immunol.</u> 1017, 1994.

The methods of protein transfer of the present invention also allow for the delivery of one or more MHC allelic variants to an APC.

In a related invention, protein transfer can also be applied to an immunomodulatory polypeptide other than a MHC polypeptide for purposes of APC engineering. In this case, the objective is generally to modulate the immunogenic potential of the APC in order to enhance its therapeutic efficacy. An immunomodulatory polypeptide, such as a cell surface costimulator, is modified through genetic or other types of manipulation to confer to it the property of membrane reincorporability. This artificial polypeptide, which by virtue of its modification is amenable to protein transfer, can be purified and then painted onto the surface of a cell, such as an APC.

Applicant has discovered that the immunogenicity of a tumor cell can be remarkably enhanced by expressing on the surface of that tumor cell an artificial costimulator polypeptide, such as a costimulator:GPI chimeric poly-peptide, that is amenable to protein transfer. One or more of such artificial costimulator polypeptides can be selected from known costimulators, for example, B7-1, B7-2, B7-3, intracellular adhesion molecule ICAM-1, ICAM-2, ICAM-3, LFA-3, VCAM-I, and fibronectin, and the modified costimulator can be used for tumor APC engineering. Methods that relate to tumor APC engineering can be readily generalized to other types of APCs.

The present invention discloses several membrane-binding polypeptide variants that are effective for coating tumor cells in order to enhance the immunogenicity of the cells. A preferred variant is a polypeptide including at least the extracellular domain of a costimu-lator polypeptide with a covalently linked glycosylphos-phatidylinositol (GPI) moiety. Such GPI-modified poly-

peptides can be readily generated through chimeric gene transfer technology. Moreover, GPI-modified polypeptides, once purified from cells, can be readily reincorporated into cell membranes.

5    In contrast to MHC protein transfer to APCs, costimulator delivery may allow for greater latitude in the applicable protein transfer techniques. Other chimeric polypeptide variants can be used effectively for cell coating. An example of such a polypeptide is one in which
10   the extracellular domain of a costimulator polypeptide is linked to streptavidin. This chimeric polypeptide can be used to coat tumor cells that have been pre-coated with a universal binding agent, such as a biotin:lipid conjugate. Yet another example of a polypeptide variant that can be
15   used for coating cells is one in which the extracellular domain of a costimulator polypeptide is linked to a second polypeptide. The second polypeptide can be one which binds to a receptor on the tumor cell, to specifically target the costimulator.

20   The present invention also provides an efficient method for identifying combinations of costimulator molecules that enable optimal enhancement of tumor cell immunogenicity. Protein transfer offers the significant advantage of being able to deliver multiple molecules simul-
25   taneously. Since substantial advantage is obtained by coating with multiple costimulators, protein transfer allows for the rapid deciphering of the optimal costimulator array for a given cell type.

According to yet another embodiment of the present
30   invention, costimulator coating is combined with other known methods for enhancing the immunogenicity of a cell such as a tumor cell. It is anticipated that additive or synergistic effects may be obtained by such combinatorial approaches. According to one combinatorial approach,
35   costimulator coating is applied to the same cell that has been otherwise manipulated to enhance its immunogenicity. For example, a transfected tumor cell expressing a soluble

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lymphokine, such as interleukin-4, can be coated by protein transfer with one or more membrane-binding GPI polypeptide derivatives of costimulators. Moreover, even three or more distinct approaches can be combined in the

5   same cell, e.g., costimulator coating, soluble lymphokine expression, and insulin-like growth factor inhibition. More optimal "universal" immunogenic tumor cells can be generated in this way.

According to a second combinatorial approach, cell
10  populations are combined to constitute a therapeutic cellular mixture, wherein individual cell populations have been modified by only a single approach for enhancing tumor cell immunogenicity. Any number of such mixed cell populations, including cells coated with costimulators,
15  can be coordinately administered to enhance therapeutic efficacy.

The present invention addresses the need for engineering tumor cell surfaces in complex ways to achieve selected cell surface molecular arrays. Methods are pro-
20  vided for identifying for each tumor type the optimal molecular array for maximal immunogenic potential. In turn, methods are provided for engineering primary tumor cells in clinical settings to efficiently effect said tumor phenotypes.

25      Thus, in a first aspect the present invention features a method for producing a cell having a defined MHC:nominal antigen peptide on its membrane. The method can be practiced by contacting the external portion of the cell with an externally applied nominal antigen peptide and an
30  artificial lipid-modified MHC polypeptide able to bind the nominal antigen peptide. Alternatively, the external portion of the cell can be first contacted with an externally applied artificial lipid-modified MHC polypeptide able to bind the nominal antigen peptide and
35  then contacted with the nominal antigen peptide so that the artificial lipid-modified MHC polypeptide binds the peptide. In a third variation, the nominal antigen

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peptide is first contacted with the artificial lipid-
modified MHC polypeptide able to bind the nominal antigen
peptide and the external portion of the cell is then con-
tacted with externally applied nominal antigen peptide
5   bound to the lipid-modified MHC polypeptide.

By "cell" is meant an antigen presenting cell or any
other cell which is capable of effectively interacting
with a T cell, leading to activation or inhibition of the
T cell.

10   By "defined" is meant predetermined molecular species.

By "nominal antigen peptide" is meant an oligopeptide
that is capable of binding to the antigen-binding groove
of an MHC molecule.

Many functional oligopeptides that are capable of
15   binding to the antigen-binding pockets of different
allelic (polymorphic) variants of class I and class II MHC
molecules are known to those who practice the art. For
some of the allelic MHC variants, nominal antigen peptide
sequence motifs have been determined that allow one to
20   predict whether any given oligopeptide will be capable of
binding to a given MHC polypeptide. In practicing the
present invention, one can draw upon this knowledge to
either select a functional nominal antigen peptide from
nominal antigen peptides known to bind a given MHC allelic
25   variant or else configure nominal antigen peptide candi-
dates based upon sequence motif requirements. Any oligo-
peptide of interest can be readily synthesized using a
commercial oligopeptide synthesizer and then contacted
with an MHC:GPI molecule.

30   By "contacting the external portion of the cell with
an externally applied..." is meant the exterior surface of
the cell membrane is contacted by applying externally the
nominal antigen peptide and the lipid-modified MHC
polypeptide either separately or together. This is in
35   contrast with intracellular insertion of these molecules
into the membrane, which occurs naturally or with genetic-
ally engineered molecules expressed within the cell.

10

By "artificial lipid-modified" is meant a covalent lipid modification that is not naturally present on the MHC molecule so modified; and functions so that the MHC molecule can be reincorporated into the cell membrane.

5   That is, such a molecule does not occur naturally in nature. Artificial lipid-modified includes any other type of modification that can function to reincorporate an MHC molecule into a membrane. A preferred artificial lipid modification according to the present invention is a GPI

10  modification. There is no known polymorphic MHC polypeptide that is naturally GPI-modified. However, the non-polymorphic mouse MHC molecule called Qa-2 is naturally GPI-modified. In particular, applicant provides human MHC polypeptide with a GPI-type modification. Other embodi-

15  ments of the present invention include the use of lipid-modified MHC molecules that comprise non-GPI lipid modifi-cations, for example, chemical coupling of a lipid moiety directly to the MHC polypeptide. Methods for covalently coupling lipids to polypeptides are described in the prior

20  art. Of note, the advantage of GPI modification over non-GPI lipid coupling methods is the former's site-specificity. Another advantage of the former is that the peptide:anchor link is natural and hence is less likely to be immunogenic. GPI-modified MHCs are made via chimeric gene

25  transfer in cells in which the recombinant protein can be produced in large amounts.

By "MHC molecule" is meant any molecule that functions to bind nominal antigen peptides and can interact with T-cells. It need not be a complete MHC molecule as it

30  exists in nature, rather it can be the active portion as determined by routine procedures. An MHC molecule capable of binding nominal antigen peptide and the T-cell receptor preferably consists of a heavy chain:$\beta$2m light chain heterodimer for class I MHC and an alpha:beta heterodimer

35  for class II MHC. However, in the case of class I MHC, as opposed to class II MHC, the antigen-binding pocket is made up entirely of the heavy chain polypeptide subunit.

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In practicing the present invention for purposes of acti-
vating or inhibiting T-cells with nominal antigen peptide-
specificity, but not alloantigen-specificity, it is pre-
ferred to select an MHC:GPI allelic variant that is syn-
5   geneic with the T-cells undergoing modulation.   For
example, in the case of human class I MHC, there are
usually six candidate MHC polypeptides to select from,
that is, two allelic variants for each of the HLA-A, HLA-
B, and HLA-C genetic loci.  The choice among these candi-
10  dates depends upon the therapeutic context.  For example,
in treating patients with chronic active hepatitis, there
is information available that generating T cells with
specificity for hepatitis B virus nominal antigen peptides
restricted to the HLA-A2.1 allelic variant may be bene-
15  ficial.  Consequently, in treating a chronic active hepa-
titis patient with HLA-A2.1 as one of the six class I MHC
allelic variants, one would select HLA-A2.1:GPI in
implementing the therapeutic method of the present inven-
tion.  The optimum MHC is readily determined by routine
20  procedures.

    Not all potential membrane-binding MHC molecular
derivatives will be capable of effective antigen presenta-
tion.  Effective antigen presentation requires both capa-
city to bind relevant nominal antigen peptide and capacity
25  to bind and trigger a relevant MHC-restricted nominal
antigen peptide-specific T-cell receptor.  Even in the
case of GPI-modified MHC derivatives, not all of them will
be able to fulfill both requirements.  Such MHC molecules
can be determined by routine procedure.

30      In a preferred embodiment of the present invention, a
recombinant MHC heterodimer is produced in a cell that
expresses empty MHC complexes at the cell surface, that
is, MHC complexes free of nominal antigen peptide.  This
facilitates the loading of an exogenous antigen peptide
35  onto purified recombinant MHC heterodimer, since there is
no resident peptide to be displaced.  The recombinant MHC
molecule is purified by immunoaffinity chromatography or

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standard biochemical purification procedures. In the case
of a GPI-modified MHC heterodimer, it is ready at this
stage for exogenous nominal antigen peptide loading (prior
to reincorporation into a cell) or for reincorporation
5    into a cell surface with simultaneous or subsequent
nominal antigen peptide loading. Another significant
feature of the present invention is the ability to control
the amount of MHC delivered to the cell surface. This is
a consequence of the dose-dependence of the coating
10   process.

Moreover, in the case of GPI-modified MHC, relatively
small amounts (in the picomolar range) of recombinant
protein are needed to achieve functional MHC molecular
densities. Yet another significant feature of the present
15   invention is the simplicity of the delivery process.
Since empty MHC heterodimers are being used, there is no
prerequisite for prolonged co-incubation of the MHC
molecule and the nominal antigen peptide, although the
degree of loading can be maximized by prolonging contact
20   between the two. One simply combines a purified MHC:GPI
and a nominal antigen peptide in a standard buffer, and
then either co-incubates the two for several hours prior
to adding the mixture to cells or immediately adds the
mixture to cells in a medium that can support cell via-
25   bility. The kinetics of MHC:GPI membrane reincorporation
are rapid, with detectable reincorporation within minutes.
Optimal reincorporation is achieved after one to two
hours, and is best accomplished in the absence of serum or
excess hydrophobic peptides. The coated cells are ready
30   at this stage for immunotherapeutic applications.

In a second aspect the present invention features a
method for producing a biological membrane having a
defined MHC:nominal antigen peptide on its surface. The
method can be practiced by contacting the biological mem-
35   brane with a nominal antigen peptide and an artificial
lipid-modified MHC polypeptide able to bind the nominal
antigen peptide. Alternatively, the biological membrane

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can be first contacted with an artificial lipid-modified
MHC polypeptide able to bind the nominal antigen peptide
and second contacted with the nominal antigen peptide so
that the artificial lipid-modified MHC polypeptide binds

5   the peptide.   In a third variation, the nominal antigen
peptide is first contacted with the artificial lipid-
modified MHC polypeptide able to bind the nominal antigen
peptide and the biological membrane is then contacted with
the nominal antigen peptide bound to the lipid-modified

10  MHC polypeptide.

A "biological membrane" can be either isolated from a
cell or artificially produced which is capable of
interacting with a T-cell, and activating or inhibiting
the T-cell.  Methods for preparing membrane extracts from

15  cells are numerous and well-known to those familiar with
the art.  For example, sonication and differential ultra-
centrifugation can be combined to prepare enriched mem-
brane fractions.  Similarly, methods for producing biolog-
ical membranes de novo are widely known, for example, com-

20  bining lipid and non-lipid components to generate lipo-
somes.    Lipid-modified MHC molecules, such as a GPI-
modified MHC molecule, can be incorporated into membrane
extracts or liposomal membranes by simply combining the
two in a standard buffer.  Alternatively, one can incor-

25  porate a lipid-modified MHC molecule into a cell surface
prior to preparing a membrane extract from that cell.
Also, a lipid-modified MHC molecule can be combined with
the other ingredients in the process of liposome forma-
tion.  Optimizing the liposome formation process in this

30  context for any given MHC:GPI is a straightforward pro-
cess.

By "membrane extract" is meant an extract of a cell
enriched for membranes, but not necessarily containing
only membranes.  Such an extract is chosen because it will

35  have the immunogenic properties necessary to induce an
immune response _in vivo_ or _ex vivo_.  By "membrane" is
meant a sheet, usually about 10 nm thick and normally com-

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posed of a bimolecular layer of lipid and protein, enclo-
sing or partially enclosing a cell, organelle, or vacuole.

In preferred embodiments of these two aspects of the
invention the MHC polypeptide comprises a class I MHC
5    polypeptide sequence; the MHC polypeptide comprises a
class II MHC polypeptide sequence; the lipid-modified MHC
polypeptide comprises a glycosyl-phosphatidylinositol-
modified MHC polypeptide; the lipid-modified MHC polypep-
tide comprises a poly-histidine tag, epitope tag or other
10   appended amino acid sequence designed to simplify purifi-
cation or detection of said polypeptide; the glycosyl-
phosphatidylinositol-modified MHC polypeptide is produced
by gene transfer of a chimeric gene expression construct
comprising a GPI modification signal into a host cell and
15   isolation of the glycosyl-phosphatidylinositol-modified
MHC polypeptide from the host cell.

By "class I MHC polypeptide sequence" is meant an
amino acid sequence corresponding to a portion of the
extracellular domain of a class I major histocompatibility
20   complex heavy chain, for example, an HLA-A, HLA-B, or HLA-
C heavy chain.

By "class II MHC polypeptide sequence " is meant an
amino acid sequence corresponding to a portion of the
extracellular domain of either a class II major histocom-
25   patibility complex alpha chain or a class II major histo-
compatibility complex beta chain, for example, an HLA-DQ,
HLA-DR, or HLA-DP alpha or beta chain.

By "glycosyl-phosphatidylinositol-modified MHC peptide
is meant an MHC polypeptide which has a covalently
30   attached glycosyl-phosphatidylinositol molecule, so as to
allow membrane insertion.

By "poly-histidine tag" is meant two or more clustered
histidines that are inserted into a polypeptide sequence
in order to permit purification of said polypeptide by
35   nickel-sepharose chromatography.   In the case of a
glycosyl-phosphatidylinositol-modified    polypeptide,
Applicant has discovered that an optimal site for inser-

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tion of the polyhistidine amino acid sequence is in be-
tween the sequence for the polypeptide of interest, for
example, an MHC polypeptide sequence, and the GPI modifi-
cation signal sequence.  In the case of GPI-modified poly-
5   peptides, the N-terminal signal peptide and C-terminal GPI
moiety preclude polyhistidine insertion into the conven-
tional N-terminal and C-terminal sites.  Insertion of the
polyhistidine sequence is accomplished by inserting the
coding sequence for this polyhistidine sequence into the
10  desired site of an MHC expression construct.

By "chimeric gene expression construct" is meant an
expression vector comprising a coding sequence comprising
two coding sequences linked in-frame that are not natu-
rally linked to each other.

15      By "GPI modification signal" is meant an amino acid
sequence, generally derived from a natural GPI-modified
polypeptide, that upon artificial insertion into another
polypeptide results in GPI modification of said second
polypeptide.

20      By "host cell" is meant a cell that is capable of
expressing a transfected gene.

By "isolation" is meant purification of the glycosyl-
phosphatidylinositol-modified MHC polypeptide from the
host cell.  Possible purification methods include affinity
25  chromatography,    ion-exchange    chromatography,    size-
exclusion chromatography, hydrophobic chromatography, and
salting-out procedures.

In a third aspect the invention features a method for
activating an antigen-specific T-cell comprising the step
30  of contacting a cell having a defined MHC: nominal antigen
peptide complex on its membrane with a T-cell population
comprising a T-cell with specificity for said MHC:nominal
antigen peptide complex.

By "activating an antigen-specific T cell" is meant
35  stimulating a T cell in a manner that results in T cell
proliferation or triggering of T cell cytotoxicity or
triggering T cell cytokine production, for example,

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interleukin-2 and interleukin-2 receptor production.
Generally, it is believed that in order to activate a T
cell, two signals comprising an antigen-specific signal,
for example, a signal delivered by an MHC:nominal antigen
5    peptide complex, and a non-specific signal, for example,
a signal delivered by a cell surface costimulator, must be
conveyed to the T cell.

By "contacting" is meant bringing one cell into
proximity with a second cell in a way that permits the
10   first cell to modulate the second cell.  In the present
invention, the contacting step can be effected ex vivo or
in vivo.  According to the present invention, an antigen-
presenting cell exogenously coated with a lipid-modified
MHC:nominal antigen peptide complex is contacted with a T-
15   cell to induce activation of said T-cell.  Methods for
contacting antigen-presenting cells with T-cells in order
to induce activation of said T-cells ex vivo or in vivo
are described extensively in the literature.  These stan-
dard T-cell activation methods can be directly applied in
20   the use of lipid-modified MHC:nominal antigen peptide com-
plexes.  Numbers of cells to be used, time of incubation,
and other conditions are well known to those familiar with
the art, as are methods for optimizing stimulation condi-
tions and tailoring them for specific antigenic systems
25   and clinical therapies.

By "T-cell with specificity for the MHC:nominal
antigen peptide complex "is meant a T-cell that bears a T-
cell receptor that can bind an MHC:nominal antigen peptide
complex in a manner that leads to T-cell activation.

30   In preferred embodiments contacting is performed _ex_
_vivo_; contacting is performed _in_ _vivo_; the MHC polypeptide
is syngeneic with said antigen-specific T-cell; the MHC
polypeptide is allogeneic with said antigen-specific T-
cell; the cell is a dendritic cell; the cell is an acti-
35   vated B-cell; the cell is another cell that bears a costim-
ulator and is thereby capable of activating an antigen-
specific T-cell; the cell is presenting a tumor antigen.

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By "ex vivo" is meant outside of the body of a patient to be treated. A preferred embodiment of the present invention is the use of an antigen-presenting cell coated with a lipid-modified MHC:nominal antigen peptide complex

5  for purposes of producing large numbers of antigen-specific T-cells. Such T-cells can then be administered to patients as a cellular therapeutic. Patients in need of such therapeutic T-cells include ones who suffer from viral diseases, cancer, and immunodeficiency disorders.

10  For instance, it is recognized that clinical progression to chronic active hepatitis in patients infected with hepatitis B virus may result from a deficiency in certain T-cells with antigen-specificity for defined hepatitis B virus nominal antigen peptides. Such patients are good

15  candidates for ex vivo therapy with antigen-specific T-cells. To treat a patient in need of therapeutic T-cells, antigen-presenting cells, such as dendritic cells, are first recovered from the patient's peripheral blood. In the case of dendritic cells, their numbers are amplified

20  ex vivo by adding cytokines, for example, a combination of GM-CSF and interleukin-4 as described (Romani et al., 180 J. Exp. Med. 83, 1994). A preparation consisting of an MHC:GPI:nominal antigen peptide comprising MHC and nominal antigen peptide components that are relevant to the

25  patient being treated, for example, in the case of a chronic active hepatitis patient bearing the common HLA-A2.1 allele, a combination of HLA-A2.1 and hepatitis B virus peptides as disclosed in the example below, are added to the cells in culture. T-cells from the patient

30  are then co-cultured with the MHC:GPI: nominal antigen peptide-coated antigen-presenting cells in order to amplify the numbers of antigen-specific T-cells. The latter cells are then administered to the patient in need of such therapeutic T-cells.

35  By "in vivo" is meant in the body of a patient to be treated. A preferred embodiment of the present invention is the use of an antigen-presenting cell coated with a

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lipid-modified MHC:nominal antigen peptide complex for purposes of inducing the proliferation of antigen-specific antigen-presenting cells in a patient. The modified antigen-presenting cells are administered directly to a
5   patient as a cellular therapeutic. Patients in need of such therapeutic T-cells include ones who suffer from viral diseases, cancer, and immunodeficiency disorders. For instance, it is recognized that T-cell anti-tumor responses can be beneficial to a patient who suffers from
10   a cancer. For certain cancer types, there is information available as to specific tumor antigen peptides, with well-defined MHC allelic restriction, that can elicit T-cell antitumor responses, for example, an HLA-A2.1-restricted melanoma nominal antigen peptide. Hence, to
15   treat such a patient, a preferred method is to isolate and amplify dendritic antigen-presenting cells from said patient's peripheral blood, coat the cells with the relevant MHC:GPI:nominal antigen peptide complex, and administer the modified antigen-presenting cells back to the
20   patient. These modified antigen-presenting cells function as a cellular immunogen in the patient to generate antigen-specific T-cells with therapeutic benefit.

By "syngeneic" is meant that the antigen-specific T-cell bears at least one MHC allelic variant that is
25   identical to that of the MHC molecule that is being transferred.

By "allogeneic" is meant that the antigen-specific T-cell does not bear any MHC allelic variants that are identical to that of the MHC molecule that is being
30   transferred.

By "dendritic cell " is meant a well-described cell generally derived from peripheral blood that has potent antigen-presenting capacity and grows with a dendritic morphology in culture.

35   By "activated B-cell" is meant a B-cell that has been triggered with a polyclonal stimulator, for example, lipopolysaccharide.

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By "presenting a tumor antigen" is meant bearing a nominal antigen peptide corresponding to a tumor-specific polypeptide.

In a fourth aspect the invention features a method for
5    inhibiting an antigen-specific T-cell comprising the step of contacting a cell having a defined MHC: nominal antigen peptide complex on its membrane with a T-cell population comprising a T-cell with specificity for said MHC:nominal antigen peptide complex.

10   By "inhibiting" is meant modulating a T-cell in a manner that results in the inability of said T-cell to proliferate or to undergo triggering of T-cell cytotoxicity or to undergo triggering of T-cell cytokine production, for example, interleukin-2 and interleukin-2 recep-
15   tor production. A modulated T-cell in such an inactive state can be either apoptotic (undergoing programmed cell death) or anergic (unresponsive).

In preferred embodiments contacting is performed ex vivo; contacting is performed in vivo; the MHC polypeptide
20   is syngeneic with said antigen-specific T-cell; the MHC polypeptide is allogeneic with said antigen-specific T-cell; the cell is expressing a coinhibitor; the cell is lacking a costimulator.

By "expressing a coinhibitor" is meant that a cell
25   surface molecule capable of delivering an inhibitory signal to a T-cell, for example, the coinhibitor CD8, is present on the cell surface. Applicant has previously established that an antigen-presenting cell can be converted from a T-cell activator to a T-cell inhibitor by
30   expressing on said antigen-presenting cell a cell surface coinhibitor such as CD8. This modified antigen-presenting cell is referred to by Applicant as an "artificial veto cell". Furthermore, it is believed that an artificial veto cell comprising the CD8 coinhibitor triggers apop-
35   tosis in antigen-specific T-cells. Thus, the present invention provides a means for producing artificial veto cells with well-defined antigenic properties. By coordi-

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nately coating a cell with a lipid-modified MHC:nominal antigen peptide complex and a coinhibitor, an artificial veto cell can be produced which is capable of deleting T-cells with specificity for said MHC-restricted nominal antigen peptide. When a syngeneic MHC polypeptide is
5   used, the method can be applied for the treatment of an autoimmune disorder. Through the use of an allogeneic MHC polypeptide, this method can be used for inhibiting an allogeneic response for the treatment of an alloimmune
10  disorder.

By "lacking a costimulator" is meant that a cell surface molecule capable of delivering an activating signal to a T-cell, for example, the costimulators B7-1, B7-2, B7-3, ICAM-1, is absent from the cell surface.
15  Generally, it is believed that in order to activate a T cell, two signals comprising an antigen-specific signal, for example, a signal delivered by an MHC:nominal antigen peptide complex, and a non-specific signal, for example, a signal delivered by a cell surface costimulator, must be
20  conveyed to the T cell. Antigen presentation in the absence of requisite costimulation leads to the induction of T-cell anergy. Thus, the present invention provides a method for producing a new category of artificial veto cells that are capable of inducing anergy in antigen-
25  specific T-cells. A critical aspect of the present invention is that it bypasses the need to use a cell that is able to process antigens on its own. This implies that essentially any cell can be used as a vehicle to present antigen. In turn, for purposes of T-cell inhibition, it
30  is therefore possible to avoid conventional antigen-presenting cells which generally express costimulators and instead select other cells which are naturally costimu-lator negative. By coating such a cell with a lipid-modified MHC:nominal antigen peptide complex, one can
35  generate an artificial veto cell. Three candidate cell types for this purpose are hepatocytes, fibroblasts, and erythrocytes.

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In a fifth aspect the invention features a glycosyl-phosphatidylinositol-modified polypeptide which is separate from a cell membrane.

By "separate from a cell membrane" means not
5  incorporated in a cell membrane and located external to a cell, as opposed to contained internally.

In a sixth aspect the invention features a cell having a membrane exogenously coated with lipid-modified MHC polypeptide.

10  Exogenous coating of the cell may result in a MHC polypeptide with a cell surface topology (e.g. association with proteins) that is distinct from a cell that has been genetically engineered to contain a MHC polypeptide on its surface (insertion into the membrane via an intracellular
15  route).

In a seventh aspect the invention features a modified antigen-presenting cell comprising a cell with an artificial costimulator polypeptide exogenously reincor-porated into the surface of the cell.

20  By "modified antigen-presenting cell" is meant an antigen-presenting cell that has a non-natural molecular moiety on its surface. For example, such a cell may not naturally have such a costimulator on its surface or may have additional artificial costimulator in addition to
25  natural costimulator on its surface.

By "artificial costimulator polypeptide" is meant a molecule that functions as a costimulator and has the capacity to be exogenously reincorporated into a cell membrane. Such a molecule may include less than an entire
30  naturally occurring costimulator molecule, or be a varia-tion on a naturally occurring costimulator molecule, but still retains the ability to act as a costimulator. Such polypeptides can be determined by routine procedures.

By "exogenously reincorporated" is meant the external
35  portion of the cell membrane is contacted with the artificial costimulator molecule such that it becomes bound to that surface. This is in contrast to intracel-

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lular insertion of these molecules into the membrane, which occurs naturally or with genetically engineered molecules expressed within the cell.

In preferred embodiments the modified antigen-presenting cell is an immunogenic tumor cell; the artificial costimulator polypeptide comprises a glycosylphosphatidylinositol moiety; artificial costimulator polypeptide comprises a streptavidin moiety; artificial costimulator polypeptide comprises a molecule with affinity for a molecule on the surface of the cell; the modified antigen-presenting cell is pre-coated with a molecule with affinity for an artificial costimulator polypeptide; the costimulator portion of the artificial costimulator polypeptide is selected from the group consisting of B7-1, B7-2, B7-3, intracellular adhesion molecule-1, intracellular adhesion molecule-2, intracellular adhesion molecule-3, LFA-3, VCAM-1, and fibronectin.

By "immunogenic tumor cell" is meant a modified tumor cell such that the tumor cell is now immunogenic. Most naturally occurring tumor cells are not immunogenic.

By "glycosyl-phosphatidylinositol moiety" is meant a GPI molecule attached to the costimulator polypeptide. The GPI moiety can be attached to the costimulator as described for an MHC molecule.

By "strepavidin moiety" is meant any molecule or portion thereof able to bind avidin. Attachment of strepavidin to a polypeptide is performed by standard procedures.

By "a molecule with affinity for a molecule on the cell surface" is meant a molecule which allows for the binding of the artificial costimulator to the cell surface. Such a molecule could be a second polypeptide that binds a receptor of the cell surface. Linkage of the second polypeptide to the costimulator could be by standard procedures such as the use of a hybrid sequence encoding both molecules.

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In a eighth aspect the invention features a method for producing a modified antigen presenting cell comprising a costimulator on its membrane. The method is practiced by contacting the external portion of the cell with an
5  externally applied artificial costimulator polypeptide.

In preferred embodiments the artificial costimulator polypeptide comprises a glycosyl-phosphatidylinositol moiety; the costimulator portion of said artificial costimulator polypeptide is selected from the group con-
10  sisting of B7-1, B7-2, B7-3, intracellular adhesion molecule-1, intracellular adhesion molecule-2, intracellular adhesion molecule-3, LFA-3, VCAM-1, and fibronectin.

In a ninth aspect the invention features a method for enhancing a cell's immunogenicity comprising contacting
15  the cell with a membrane-binding artificial costimulator polypeptide.

By "membrane-binding" is meant the polypeptide has the capacity to noncovalently or covalently attach to a biological membrane.
20  In an tenth aspect the invention features a method for inducing anti-tumor immunity against an autologous tumor cell in a patient by administering to the patient a tumor cell or tumor cell membrane extract with externally reincorporated costimulator polypeptide on its membrane.
25  By "autologous tumor cell" is meant a tumor cell of the same tumor type that is derived from the patient undergoing treatment.

By "tumor cell membrane extract" is meant a membrane extract, as defined above, derived from a tumor cell.
30  In preferred embodiments the tumor cell is an autologous tumor cell, the tumor cell is a heterologous tumor cell.

By "heterologous tumor cell" is meant a tumor cell of the same tumor type that is derived from an individual
35  other than the patient.

In a eleventh aspect the invention features a method for identifying a molecule that enhances the immuno-

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genicity of an antigen presenting cell.  The method encompasses coating the antigen presenting cell with a membrane binding polypeptide derivative of the molecule and determining the immunogenicity of the resulting cell
5  compared to an uncoated cell.

By "coating" is meant exogenously incorporating a polypeptide into the external aspect of a cell membrane.

By "enhancing immunogenicity" is meant increasing the immunogenic potential of a cell.
10     In preferred embodiments the antigen presenting cell is a tumor cell; determining the immunogenicity comprises determining the capacity of the coated tumor cell to induce an antitumor immune response.

By "antitumor immune response" is meant an
15  immunological cell-mediated or antibody-mediated response directed against a tumor cell.

A significant aspect of this invention is the discovery that protein transfer can be used to deliver a functional MHC polypeptide or costimulator polypeptide to
20  a cell surface.  Previously only gene transfer was used for this purpose.  The use of protein transfer for effecting said delivery process provides substantial advantages for engineering therapeutic APCs, since it bypasses the significant limitations imposed by those delivery methods
25  based upon gene transfer.  Advantages of protein transfer over gene transfer for APC engineering include, but are not limited to, the following.

First, most APC candidates, by virtue of being primary cells (that is, nontransformed) with low proliferative
30  rates, are not amenable to efficient gene transfer.  In contrast, primary cells can readily be coated with exogenously added artificial polypeptides designed for efficient membrane reincorporation and cell surface coating.  Thus, the present invention can be practiced
35  with a greater variety of cells, and most importantly with cells that are APC candidates.

25

Second, nucleic acid, such as DNA, that is exogenously introduced into cells is often unstable, with concomitant loss of expression of the encoded RNA product. In contrast, protein that is exogenously introduced into
5   cellular membranes has defined stability characteristics.

Third, transfecting the same cell with multiple genetic constructs is a cumbersome process. In fact, from a practical standpoint, cotransfecting more than two genes into even a transfectable cell constitutes a major under-
10  taking. Hence, this approach is not well-suited for the clinical setting, where many samples of often difficult to transfect cells are to be processed. In contrast, protein transfer allows for the simultaneous delivery of any number of membrane-binding polypeptides to the cell sur-
15  face, with no additional burden imposed when additional molecules are to be included. The latter simply requires the addition of more molecules into the mixture. Hence, multiple MHC:nominal antigen peptide complexes or costimu- lators can be coordinately delivered to the same cell by
20  protein transfer.

Fourth, certain gene transfer-based immunotherapeutic strategies require selection for stable transfectants. This can be a time consuming process and complicates the clinical practice of such methods and imposes a delay
25  period between biopsy and treatment. In contrast, protein transfer is a relatively rapid process and does not impose a burden of excessive cell culturing, shortening the interval between obtaining of cells from the patient and treatment.

30  Fifth, there is considerable variability in the number of molecules that are expressed at the cell surface of transfected cells. In contrast, protein transfer provides for fine control of the degree of coating, with the possibility of either very low or high levels of protein
35  delivery to the cell surface, and more uniformity with respect to the number of molecules on individual cells of the population.

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Sixth, gene transfer is dependent in most instances upon genetic vectors comprising viral components which carry with them some degree, albeit small, of biosafety hazard. In contrast, no biosafety hazards are associated

5  with polypeptide derivatives to be used for practicing protein transfer onto APCs. The polypeptide derivatives of the present invention are designed to incorporate molecular components that are, in most instances, derived from native polypeptides, and hence, low toxicity and

10  immunogenicity constitute a fundamental feature of such polypeptide derivatives.

The method of the present invention also overcomes several of the limitations of the conventional method of loading oligopeptides onto APCs.

15  First, in order to replace pre-engaged, endogenously-loaded peptides from the antigen binding grooves of MHC molecules, in particular class I MHC molecules, by _in situ_ loading at the cell surface, a large excess of exogenous peptide is required. Under such circumstances, there is

20  a lack of fine control of the final density of any given MHC:nominal antigen peptide complex at the cell surface. This represents a serious limitation since there is evidence that MHC:nominal antigen peptide complex density can dictate the balance between T-cell activation and

25  inhibition. Second, certain peptides cannot be efficiently loaded at all. Third, one is restricted to the use of cells already bearing relevant MHC molecules and to the levels of specific MHC products that naturally occur at the cell surface.

30  The protein transfer method of the claimed invention allows for the coating of any cell with any relevant MHC molecule and any nominal antigen peptide that can bind to that MHC molecule. Also, the method allows for fine control of the MHC:nominal antigen peptide complex density

35  on the surface of an APC as the levels of various reagents can be precisely controlled.

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Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

5      The drawings will first briefly be described.

Figure 1 is a schematic drawing of expression constructs used for HLA-A2.1:GPI/$\beta_2$m and HLA-A2.1/$\beta_2$m heterodimers in Schneider S2 cells.

Figure 2 is a silver-stained SDS-PAGE gel which 10 documents the identity of purified HLA-A2.1:GPI/$\beta_2$m and HLA-A2.1/$\beta_2$m heterodimers. Designations to the right correspond with the HLA-A2.1 heavy chain (HLA-A2.1), HLA-A2.1:GPI heavy chain (HLA-A2.1:GPI), and human $\beta_2$m ($\beta_2$m).

Figure 3 is a bar graph plotting data from a sandwich 15 ELISA which documents heterodimerization in both purified HLA-A2.1:GPI/$\beta_2$m and HLA-A2.1/$\beta_2$m. Differing quantities of HLA-A2.1/$\beta_2$m (cross-hatched boxes) and HLA-A2.1:GPI/$\beta_2$m (filled boxes) heterodimers bound to PA2.1 antibody pre-coated microtiter wells are shown on the x-axis. HRP 20 activity from bound horseradish peroxidase-conjugated goat anti-human $\beta_2$m as measured by absorbance at 490 nm is shown on the y-axis.

Figure 4 are tracings from flow cytometric and immunofluorescence analyses that compare protein transfer 25 of empty HLA-A2.1:GPI/$\beta_2$m heterodimers under different experimental conditions. The data are plotted as number of cells (y-axis) versus log fluorescence intensity (x-axis). Figure 4A shows the time course of HLA-A2.1:GPI/$\beta_2$m protein transfer. Incubation time were 0 minutes (light 30 solid line), 1 minute (long broken line), 10 minutes (medium broken line), 30 minutes (short broken line) and 60 minutes (heavy solid line). Figure 4B shows the dose-dependence of HLA-A2.1: GPI/$\beta_2$m protein transfer. Amounts of HLA-A2.1:GPI/$\beta_2$m are: 0 pmoles (light solid line); 0.1 35 pmoles (short broken line); 0.2 pmoles (medium broken line); and 1 pmole (heavy solid line). Figure 4C docu-

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ments that PI-PLC treatment releases protein-transferred
HLA-A2.1:GPI/$\beta_2$m from the cell surface. Coated cells were
incubated at 4°C or 37°C ± PI-PLC: C1R + PI-PLC (light
solid line); C1R + HLA-A2.1:GPI + PI-PLC, 37°C (medium
5    broken line); C1R + HLA-A2.1:GPI, 37°C (short broken
line); and C1R + HLA-A2.1:GPI, 4°C (heavy solid line).
Figure 4D shows the effect of loaded peptide upon HLA-A2.-
1:GPI/$\beta_2$m protein transfer: C1R (light solid line); C1R +
HLA-A2.1:GPI (heavy solid line); C1R + HLA-A2.1:GPI +
10   MA58-66 (short broken line); and C1R + HLA-A2.1:GPI + HBV
env 335-343 (medium broken line).

        Figures 5 consists of bar graph plotting data that
document    CTL    recognition    of    protein-transferred
HLA-A2.1:GPI/$\beta_2$m/peptide  heterotrimeric  complexes.
15   Percent specific lysis is plotted on the x-axis. Femtomo-
les of HLA-A2.1:GPI/$\beta_2$m or HLA-A2.1/$\beta_2$m, plus or minus
peptide (MA58-66 or HBV env 335-343) are indicated on the
y-axis. Error bars correspond to one standard deviation.

        Figure 6 consists of a bar graph that compares
20   different HLA-A2.1:GPI/$\beta_2$m peptide loading regimens (either
during a pre-incubation period or during protein transfer
only). Cytotoxicity was assessed without (left) or with
(right) 1.25 $\mu$g/Ml HBV env335-343 peptide added to C1R
targets during the CTL assay. The x-axis indicates per-
25   cent specific lysis. The y-axis indicates the loading
regimens.    Error  bars  correspond  to  one  standard
deviation.

        Figure 7 consists of a graph plotting data from a
cytotoxicity assay demonstrating that artificial GPI-
30   modified B7-1 functions well as a costimulator. Various
effector cells were used: 1 EL4 (solid square); 2 EL4
(solid circle); 3 MB7-DS/EL4 (solid triangle); and 4 MB7-
DS/EL4 (solid diamond). Effector- to-target (E:T) ratios
are shown on the x-axis. Percent specific lysis is plot-
35   ted on the y-axis. Error bars correspond to one standard
deviation.

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## Description of the Preferred Embodiment

## Protein transfer of MHC:GPI complexes to cell surfaces

The present invention addresses the need for delivering antigenic complexes in a controlled fashion to
5 the surfaces of diverse cell types. Methods are provided for coating cells with lipid-modified MHC:nominal antigen peptide complexes.

In the experiments illustrated by the following examples, recombinant glycosyl-phosphatidylinositol (GPI)-
10 modified HLA-A2.1 (HLA-A2.1:GPI/$\beta_2$m) was used as a protein transfer vehicle to deliver a hepatitis B virus (HBV) nominal antigen peptide to the surfaces of cytotoxic T-cell targets. The model system utilized an HLA-A2.1-restricted hepatitis B virus (HBV) peptide and well-
15 defined human T-cell clones with specificity for this peptide. This peptide is one of a subset of HBV peptides that previous studies have defined as being dominant in T-cell responses to HBV. Clinical correlations have suggested that lack of CTL responses to these HBV peptide
20 determinants may be an important factor in clinical pro-gression to chronic active hepatitis (Penna et al., 174 J. Exp. Med. 1565, 1991; Nayersina et al., 150 J. Immunol. 4659, 1993).

Empty HLA-A2.1:GPI/$\beta_2$m was first produced in D.
25 melanogaster co-transfectants and immunoaffinity purified. Cell coating with HLA-A2.1:GPI/$\beta_2$m was shown to occur rapidly, and to be protein concentration-dependent. Protein-transferred HLA-A2.1:GPI/$\beta_2$m effectively presented an HBV peptide to peptide-specific, HLA-A2.1-restricted
30 T-cell clones in cytotoxicity assays. Protein transfer of GPI-modified class I MHC:nominal antigen peptide complexes represent a novel strategy for delivering functional anti-genic complexes to cell surfaces which bypasses limita-tions of gene transfer and permits control of nominal
35 antigen peptide densities at cell surfaces.

30

Example 1:    Production of GPI-modified Human Class I
              MHC

In order to produce HLA-A2.1 polypeptides devoid of
nominal antigen peptides, expression was performed in
5  Schneider S2 *D. melanogaster* cells. Schneider S2 cells,
obtained from J. Incardona (Case Western Reserve Universi-
ty), were maintained in 67.5% M3 medium (Sigma) supple-
mented with 20% 5X BPYE (12.5 g/l bacto-peptone, 5 g/l TC
yeastolate), 12.5% fetal bovine serum, and 20 U/ml
10  penicillin, and 20 mg/ml streptomycin. Such cells have
been shown to produce empty (nominal antigen peptide-
free), native HLA-A2.1 when cotransfected with HLA-A2.1
and human $\beta_2$m coding sequences (Jackson et al., 89 Proc.
Natl. Acad. Sci. USA 12117, 1992; Jackson et al., 263
15  Science 384, 1994). Moreover, these cells are known to
support GPI-modification (Hortsch and Goodman, 265 J.
Biol. Chem. 15104, 1990). To produce HLA-A2.1:GPI,
Schneider S2 cells were cotransfected by lipofection with
a combination of three expression constructs (Figure 1),
20  pHLA-A2.1:DAF-S /Pac (encoding HLA-A2.1:GPI heavy chain),
ph$\beta_2$m/Pac (encoding human $\beta_2$m) and pHph/Pac (encoding the
selectable marker *hph* which confers hygromycin B- resis-
tance). Alternatively, to produce native HLA-A2.1, stable
cotransfectants were generated in which pHLA- A2.1/Pac
25  (encoding native HLA-A2.1 heavy chain) was substituted for
pHLA-A2.1:GPI/Pac in the above combination of expression
constructs.

The HLA-A2.1:GPI heavy chain and HLA-A2.1 heavy chain,
and $\beta_2$m expression constructs were produced by ligating
30  coding sequences for HLA-A2.1, HLA-A2.1:DAF-S, and human
$\beta_2$-microglobulin into the *D. melanogaster* actin 5C
promoter-based expression vector, pPac (Krasnow et al., 57
Cell 1031, 1989). DNA inserts for HLA-A2.1, HLA-A2.1:
GPI, and $\beta_2$m were excised from pHLA-A2.1/REP7$\beta$ with Xho I,
35  from pHLA-A2.1:DAF-S/REP7$\beta$ (Huang et al., 31 Mol. Immunol.
1017, 1994) with Xho I and Bam HI, and ph$\beta_2$m/REP10 (Huang
et al., 31 Mol. Immunol. 1017, 1994) by BamHI and Hind III

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digestion, respectively.    Insert DNAs were blunted by
Klenow fill-in reactions and ligated into the Klenow
filled-in BamHI site of pPac.

    S2 cells were co-transfected as follows:    30 $\mu$g of
5  lipofectin (BRL), 10 $\mu$g of Qiagen column-purified
expression construct (a 1:1 mixture of ph$\beta_2$m/Pac plus
either pHLA-A2.1:DAF-S/Pac or pHLA-A2.1/Pac), and 1 $\mu$g of
pHph/Pac, to allow for selection by growth in hygromycin
B, were combined with $10^6$ washed S2 cells in 1 mL of 1.25X
10  M3 medium (Sigma) in polystyrene tubes.    After 3 hours at
room temperature, cells were washed with 10 mL complete M3
medium (67.5 % 1.25X M3 media, 20% 5X BPYE (12.5 g/L
bacto-peptone/5 g/L yeast extract), and 12.5% fetal bovine
serum and resuspended in 10 mL of complete medium.    After
15  three days, selection was initiated by adding hygromycin
B (CalBiochem) to 0.1 mg/ml with fresh selective medium
which was replaced every 3-4 days.    Transfected cell
colonies were evident at three weeks and were cloned from
soft agar.  Transfectant cell lines were screened for
20  expression of cell surface HLA-A2.1 epitopes by immuno-
staining with the HLA-A2.1, -Aw68-specific monoclonal
antibody, PA2.1 (hybridoma cell line purchased from the
American Type Culture Collection), followed by FITC-
conjugated goat anti-mouse immunoglobulin and analysis by
25  flow cytometry (FACSCAN, Becton-Dickinson).    GPI- modifi-
cation of HLA-A2.1:GPI was confirmed by treatment of
transfected cells with 10 units of PI-PLC prior to PA2.1
immunostaining as described (Huang et al., 31 <u>Mol. Immuno-</u>
<u>l.</u> 1017, 1994).

30    For quantitative production of HLA-A2.1:GPI/$\beta_2$m and
HLA-A2.1/$\beta_2$m, 2-5 X $10^{10}$ S2 transfectant cells from 8-16 L
of culture were processed batchwise for immunoaffinity
purification by PA2.1-conjugated Sepharose CL-4B chromato-
graphy (Parham, 92 <u>Meth. Enzymol.</u> 110, 1983).    Elution
35  fractions containing heterodimer were identified using
both SDS-PAGE analysis and antigen-capture ELISA specific
for HLA-A2.1.    Typical yields were approximately 1 to 10

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μg recombinant HLA-A2.1:GPI/$\beta_2$m or HLA-A2.1/$\beta_2$m heterodimer
per batch with purity estimated at 60% by scanning densi-
tometry.  Neutralized immunoaffinity chromatography frac-
tions were used directly in assays.

5       Empty HLA-A2.1:GPI or HLA-A2.1, each complexed with
$\beta_2$m, were immunoaffinity purified using the HLA-A2.1 heavy
chain-specific monoclonal antibody PA2.1 conjugated to
sepharose CL-4B (Parham, 92 Methods Enzymol. 110, 1983).
The association of $\beta_2$m with either HLA-A2.1:GPI or HLA-A2.1
10  heavy    chains    in    respective    cotransfectants    was
demonstrated by subjecting immunoaffinity-purified materi-
al to SDS-PAGE analysis (Figure 2).  Approximately 1 ng of
HLA-A2.1 (lane 1) and HLA-A2.1:GPI (lane 2) heterodimers,
immunoaffinity purified using the monoclonal antibody
15  PA2.1, were separated on reducing, 15% acrylamide SDS-PAGE
gels, according to standard conditions, and visualized by
silver staining.  As can be seen in both lanes 1 and 2,
molecular species corresponding to both HLA-A2.1 or HLA-
A2.1:GPI and $\beta_2$m are present in both lanes, indicating that
20  hetorodimers were formed.

The association of $\beta_2$m with either HLA-A2.1:GPI or HLA-
A2.1 heavy chains was also demonstrated using a sandwich
ELISA (Parker et al., 29 Mol. Immunol. 371, 1992)
incorporating both anti-heavy chain and anti-$\beta_2$m antibodies
25  (Figure 3).  Molecules detected by this particular ELISA
bear both heavy chain and $\beta_2$m epitopes.

Sandwich ELISA assays were performed as follows:  1 μg
of the HLA-A2.1, -Aw68 specific monoclonal antibody,
PA2.1, was coated per well of 96 well Immulon 4 (Dynatec-
30  h) plates by overnight incubation at 4°C.  After washing
with ddH$_2$O, plates were blocked with 0.25% (w/v) bovine
serum albumin plus 0.05% (w/v) tween-20 overnight at 4°C.
Wells were washed thrice with ddH$_2$O prior to use.  Heterod-
imers were diluted into blocking buffer and captured for
35  90 minutes at room temperature.  Wells were washed thrice
with ddH$_2$O and captured antigens detected with 1:1000
diluted rabbit anti-human $\beta_2$m immunoglobulin conjugated to

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horseradish peroxidase (Accurate) and incubated at room
temperature for 30 minutes. Wells were washed thrice and
ELISA wells developed with 0.1 mL of 0.6 mg/mL ortho-phen-
yl-diamine (Sigma) in 0.1 M sodium citrate/0.01% (w/v) $H_2O_2$
5  at room temperature for 15-20 minutes. Reactions were
terminated with 2N $H_2SO_4$. Well absorbance was measured at
490 nanometers.

Example 2:    Protein Transfer of HLA-A2.1:GPI/$\beta_2$m
              Heterodimers

10        Immunoaffinity-purified HLA-A2.1:GPI/$\beta_2$m heterodimers
were evaluated for their reincorporability into cell
membranes. Co-incubation at 37°C of HLA-A2.1: GPI/$\beta_2$m with
the HLA-A-negative, HLA-B-reduced B lymphoblastoid cell
line, C1R, conferred HLA-A2.1 epitopes to C1R cell
15  surfaces. C1R cells (Storkus et al., 138 J. Immunol.
1657, 1987) were grown in RPMI-1640 supplemented with 10%
fetal bovine serum, 2 mM glutamine, and 20 U/ml penicil-
lin, and 20 mg/ml streptomycin. The transfer of HLA-A2.1:
GPI/$\beta_2$m molecules onto C1R cells was dependent upon co-
20  incubation time, temperature, HLA-A2.1:GPI/$\beta_2$m concentra-
tion, and fetal bovine serum concentration.

        To examine transfer dependence on time, washed C1R
cells were coated with HLA-A2.1:GPI/$\beta_2$m by a protein
transfer method (vide infra). Approximately 4 pmoles of
25  HLA-A2.1:GPI/ $\beta_2$m was combined with washed C1R cells at
37°C for different incubation times (0 minutes, 1 minute,
10 minutes, 30 minutes, 60 minutes), and coated cells were
then washed twice with 1xPBS/0.1% BSA/0.05 % $NaN_3$ and
immunostained with either 1 $\mu$l of PA2.1 ascites or 1 $\mu$l of
30  BB7.1 ascites as negative control. BB7.1 staining was
negative (not shown). Reincorporation was detected within
one minute of combining HLA-A2.1:GPI/$\beta_2$m with cells, and
HLA-A2.1 epitope density, as monitored by immunofluores-
cence and flow cytometry, increased steadily over a one
35  hour co-incubation period at 37°C (Figure 4A). In con-

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trast, less protein transfer was observed at either 4°C or 25°C (data not shown).

To examine transfer dependence on HLA-A2.1:GPI/ $\beta_2$m concentration washed C1R cells were incubated with varying
5   amounts (0 pmoles, 0.1 pmoles, 0.2 pmoles, 1 pmole) of HLA-A2.1:GPI/$\beta_2$m at 37°C for one hour and processed for immunostaining with PA2.1 or BB7.1 antibody.   BB7.1 staining was negative (not shown).   Increasing the HLA-A2.1:GPI/$\beta_2$m concentration resulted in a dose-dependent
10  increase in HLA-A2.1 epitope bound to C1R cell surfaces (Figure 4B).

In parallel experiments, two additional HLA- A2.1-negative cell lines, the human chronic myeloid leukemia cell line K562 and the murine thymoma cell line EL-4 were
15  comparably    coated    with    equivalent    amounts    of HLA-A2.1:GPI/$\beta_2$m (data not shown).

The influence of peptides on GPI-modified protein re-incorporation was also determined.   HLA-A2.1:GPI/$\beta_2$m was pre-incubated overnight on ice with 5 $\mu$g/mL of either
20  MA58-66 or HBV env335-343 peptide, as indicated in the legend inset.   Reactants were added to washed C1R cells, and the treated cells were immunostained with PA2.1 or BB7.1 prior to flow cytometry.   No immunostaining with BB7.1 was observed (not shown).   Short peptides at
25  concentrations below 5 $\mu$g/mL generally had no effect on the efficiency of protein transfer (Figure 4D).   However, for one peptide, MA58-66, some inhibition was observed at higher (>5 $\mu$g/mL) peptide concentrations (data not shown), perhaps an effect of its relative hydrophobicity compared
30  to the other peptides examined and potential hydrophobic interactions with the GPI anchors.

GPI    anchorage    of    reincorporated    HLA-A2.1:GPI    was substantiated by enzymatic cleavage analysis.   Four pmoles of HLA-A2.1:GPI/$\beta_2$m was protein transferred onto washed
35  C1R, cells and coated cells were then incubated at 4°C or 37°C with or without PI-PLC.   As a negative control, uncoated C1R cells were also treated with PI-PLC at 37°C.

35

Cells were processed for immunostaining with PA2.1 or BB7.1 and subsequent flow cytometry. No immunostaining with BB7.1 was detected (not shown). As expected, PI-PLC treatment of HLA-A2.1:GPI/$\beta_2$m-coated C1R cells released the

5 HLA-A2.1 epitopes from cell surfaces (Figure 4C). In addition, protein transfer of HLA-A2.1: GPI was inhibited by inclusion of 10% fetal bovine serum in the co-incubation mixture (data not shown), but once reincorporated, HLA-A2.1:GPI/$\beta_2$m could not be stripped from the

10 cell surface by washing with 10% fetal bovine serum. In contrast, insect cell-derived native HLA-A2.1/$\beta_2$m non-specifically adsorbed to cell surfaces was readily strip-ped off by this treatment (data not shown). Taken to-gether, these findings are consistent with GPI membrane

15 anchorage for HLA-A2.1:GPI/$\beta_2$m.

Example 3:     Coating of Cells With
               HLA- A2.1:GPI/$\beta_2$m/peptide

Cells were coated with HLA-A2.1:GPI molecules as follows. Approximately 20 femtomoles (~1 ng) of HLA-

20 A2.1:GPI heterodimer in 50 $\mu$l were pre-incubated with an equal volume of 1x PBS $\pm$ 0.5 $\mu$g/ml of peptide and incubated on ice overnight in a silanized microfuge tubes. Peptides were resuspended in DMSO at 20 mg/ml and diluted to 1 mg/mL in RPMI-1640 immediately prior to use. Cells

25 to be coated were washed twice with 1x PBS, resuspended at 1.25 x 10$^6$/mL in 1xPBS, and 0.25-0.5x10$^6$ cells added to the HLA-A2.1:GPI/ $\beta$2m mixtures. Final volumes were 0.25-0.5 mL. Tubes were rotated at 37°C for one hour and washed twice with 1xPBS/ 0.5% bovine serum albumin/0.1% NaN$_3$ prior

30 to staining with monoclonal antibodies for immunostaining and subsequent flow cytometry (FACSCAN, Becton-Dickinson).

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Example 4:    Cytolytic   T   Lymphocyte   Recognition   of
              Protein Transferred HLA-A2.1:GPI/$\beta_2$m/peptide
              Complexes

      The capacity of protein transferred HLA-A2.1: GPI/$\beta_2$m
5  and HLA-A2.1/$\beta_2$m to engage and present nominal antigen
   peptides was assessed in a series of functional studies.
   The   experimental   system   used   HLA-A2.1:GPI/$\beta_2$m-   or
   HLA-A2.1/$\beta_2$m-coated C1R cells as cytolytic T lymphocyte
   ("CTL") targets.

10      In a first experiment designed to test peptide
   binding, HLA-A2.1:GPI/$\beta_2$m or HLA-A2.1/$\beta_2$m heterodimers were
   pre-incubated with (or without) HLA-A2.1- restricted
   peptides overnight at 4°C prior to protein transfer.
   Under the coating conditions used in this experiment, a
15 modest level (about two-fold log fluorescence units over
   background) of cell surface HLA-A2.1 epitope was attained
   (Figure 4D).  HLA-A2.1:GPI/ $\beta_2$m + HBV envelope peptide (HBV
   env335-343)-coated, $^{51}$Cr-labeled C1R cells were efficiently
   lysed by a CD8$^+$, HBV env335-343- specific, HLA-A2.1-
20 restricted human T-cell clone (Figure 5).  HBV env335-343-
   specific T cell clones, obtained from F. Chisari, were
   derived and maintained as described (Nayersina et al., 150
   J. Immunol. 4659, 1993).  In contrast, there was no CTL
   recognition of C1R cells combined with native HLA-A2.1/$\beta_2$m
25 and the same HBV env335-343 peptide.  Similar results were
   obtained using a second distinct human CD8$^+$ T-cell clone
   with the same antigenic specificity (data not shown).
   Neither CTL clone lysed cells coated with HLA-A2.1:GPI/$\beta_2$m
   heterodimers alone or HLA-A2.1:GPI/$\beta_2$m heterodimers loaded
30 with an irrelevant HLA-A2.1-binding peptide, namely, the
   influenza matrix peptide MA58-66.  When a larger quantity
   of HLA-A2.1:GPI/$\beta_2$m/HBV env335-343 heterotrimeric complexes
   were coated onto C1R target cells, the extent of CTL-
   mediated cytolysis increased (Figure 5).

35      The specificity of CTL recognition of protein-
   transferred, peptide-loaded HLA-A2.1:GPI/$\beta_2$m was probed in
   additional experiments.    An excess of the irrelevant

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HLA-A2.1-binding peptide MA58-66, added prior to protein transfer, effectively blocked cytolysis by HBV env335-343-specific CTL of coated C1R targets (data not shown). Cytolysis was also shown to be HLA-A2.1-specific in
5   monoclonal antibody blocking studies. Both an HLA-A2.1-specific antibody (MA2.1, hybridoma cell line purchased from the American Type Culture Collection) and a pan-class I HLA-specific antibody (W6/32, hybridoma cell line purchased from the American Type Culture Collection)
10  independently blocked killing of HLA-A2.1:GPI/$\beta_2$m/HBV env335-343-coated target C1R cells. An irrelevant HLA-B7-specific monoclonal antibody (BB7.1) had no inhibitory effect (data not shown). Of note, cytolysis was also partially blocked using a human CD8$\alpha$-specific
15  antibody (Leu2a, hybridoma cell line provided by R. Evans, Roswell Park Memorial Institute), consistent with partial CD8-dependence for the CTLs.

In the above experiments, HLA-A2.1:GPI/$\beta_2$m/ peptide heterotrimers were formed in the course of an overnight
20  co-incubation at 4°C prior to protein transfer. Additional experiments were performed employing alternative heterotrimer co-incubation strategies. In these experiments, heterodimers were incubated at 4°C overnight in the absence of peptide. Peptides were subsequently
25  added during the one-hour protein transfer procedure and/or the CTL assay. As shown in Figure 6, inclusion of peptide during the protein transfer phase only was sufficient to permit significant specific CTL lysis. This is consistent with either rapid formation of MHC:peptide
30  complexes in solution during protein transfer and/or binding of peptide to reincorporated MHC heterodimers *in situ*. The latter possibility is supported by the additional finding that addition of peptide to (peptide-free) heterodimer-coated, but not uncoated, cells during the CTL
35  assay phase only also yields specific cytolysis (Figure 6).

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The extent of CTL-mediated cytolysis of target C1R cells coated with pre-formed HLA-A2.1:GPI/$\beta_2$m/peptide heterotrimeric complexes was consistently greater (by 1.25- to two-fold) than that for C1R cells coated with
5   heterodimers and later exposed to free peptide during protein transfer (Figure 6). This likely indicates that a higher proportion of loaded HLA-A2.1:GPI/$\beta_2$m/peptide heterotrimers can be achieved by prolonged overnight incubation. Moreover, it is consistent with the notion that
10  heterotrimeric complexes are forming prior to coincubation with cells. Additional evidence for the formation of pre-formed heterotrimeric complexes prior to protein transfer comes from a sandwich ELISA in which exogenous peptide was shown to increase $\beta_2$m-dependent conformational epitopes on
15  the HLA-A2.1:GPI heavy chain (data not shown).

CTL assays were performed as follows. Twice washed C1R cells were labeled with $^{51}$Cr for 1 h at 37°C, and washed twice with 1x PBS. Labeled cells were resuspended in 1x PBS and combined with the pre-incubated heterodimer
20  ± peptide in silanized microfuge tubes. Final concentrations were $10^6$ $^{51}$Cr-labeled C1R cells/mL, and unless indicated otherwise, 1 ng heterodimer/mL. Coated cells were washed twice with RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine and
25  penicillin/streptomycin. $5 \times 10^3$ coated target cells and CTL were added per well in 0.1 mL each into conical bottom microtiter wells (Linbro). For assays done in the presence of peptide, coated target cells and peptide were added in 50 $\mu$l each. CTL clone O'R875.20-44 was used at
30  an effector to target ratio of 1. Spontaneous release was measured from samples incubated with 0.1 mL of medium instead of CTL. Total release was obtained from wells treated with 0.1 mL of 4% Triton X-100. All determinations were performed in triplicate. Plates were spun for
35  2 minutes at 400 x g and incubated 4 hours at 37°C in a humidified 5% $CO_2$ incubator. Plates were centrifuged again and 0.1 mL of supernatant harvested for gamma counting.

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Percent specific release = 100*(experimental release -
spontaneous release)/(Total release - spontaneous
release).

These experiments define a novel approach for
5    delivering pre-formed class I MHC/$\beta_2$m/peptide heterotrimers
to cell surfaces that is based upon the use of GPI-
modified, membrane-reincorporable class I heavy chains.
A significant feature of the experimental system is the
use of S2 Schneider insect cells to produce empty
10   (peptide-free) HLA-A2.1, which presumably facilitates
exogenous peptide loading.

By design, relatively small quantities of HLA-
A2.1:GPI/$\beta_2$m/peptide heterotrimers were used in these
experiments for the cell coating step. The goal here was
15   to deliver limited amounts of HLA heterotrimers to cell
surfaces. The immunofluorescence data indicate that fine
control of heterotrimer densities can be achieved using
recombinant GPI-modified heterotrimers. In view of recent
data indicating that surface HLA complex density can
20   influence the balance between activation versus inhibition
during antigen presentation, such fine control is likely
to be useful. Substantially higher surface densities can
be achieved if desired by increasing the amount of GPI-
modified proteins added to the cells (data not shown).

25   The findings of the present study with HLA-A2.1 are
applicable to other class I allelic products, as well as
to class II MHC polypeptides. In the case of GPI-modified
class II MHC polypeptides, both the alpha and beta chains
are produced as GPI-modified polypeptide variants. When
30   co-expressed in the same cell via gene transfer, the two
GPI-modified polypeptide chains associate in the proper
mann r to constitute an antigen-binding pocket at the
amino-terminal ends. In order to achieve this end, it is
imperative to use similar GPI modification signal
35   sequences for both polypeptide chains so that the
molecular topology permits dimerization.

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Example 5:     Production of an artificial veto cell by
                protein transfer of an HLA-A2.1:GPI/$\beta_2$m
                peptide complex

A fundamental aspect of the present invention is the
5   capacity to deliver an MHC:nominal antigen peptide complex
to a cell that does not normally express that MHC molecule
or to a cell that does not efficiently process antigen or
to a cell that cannot be readily transfected with an MHC
gene expression construct.    Particular advantages that
10  ensue from this are especially apparent in therapeutic
applications directed towards the inhibition of pathogenic
antigen-specific T-cells.    In this case, the present
invention permits the production of highly useful
artificial veto cells that function to apoptose or aner-
15  gize antigen-specific T-cells.

Patients suffering from autoimmune or alloimmune
disorders are in need of the elimination of pathogenic T-
cells.   This can be accomplished through the use of an
artificial veto cell that targets the culprit antigen-
20  specific T-cells via the specific MHC:nominal antigen pep-
tide complex on the artificial veto cell which engages the
T-cell receptor on the T-cell.   Applicant has previously
discovered that an antigen-presenting cell can be con-
verted into an artificial veto cell by expressing the
25  coinhibitor CD8 on the antigen-presenting cell surface.
However, in that case, antigen presentation was accom-
plished by endogenous antigen processing by the artificial
veto cell.   The present invention provides a more flexible
approach in that one is not dependent upon an endogenous
30  antigen processing event, and furthermore, one can control
the amount of a uniform population MHC:nominal antigen
peptide complex at the surface of the artificial veto
cell.

According to one preferred embodiment of the present
35  invention, a costimulator-negative cell from a patient is
coated simultaneously with an MHC:GPI:nominal antigen
peptide complex and the artificial coinhibitor protein

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CD8:GPI, both of which are amenable to protein transfer. There are numerous candidate cell types, but one simple cell type to work with in this context is an autologous erythrocyte from a patient. In treating an HLA-A2.1-

5 positive patient with multiple sclerosis, one proceeds according to the following steps. Peripheral blood is obtained from the patient by venipuncture, and erythrocytes are isolated by differential centrifugation. 10 ng of HLA-A2.1:GPI, pre-incubated overnight with a

10 myelin basic protein peptide known to be pathogenic, is added together with 25 ug of CD8:GPI to the erythrocytes. The erythrocytes and recombinant lipid-modified poly-peptides are coincubated for two hours at 37C. The coated erythrocytes are then washed three times with suitable

15 buffer and then transfused back into the patient. The simplicity of this procedure stems from the fact that mem-brane reincorporation is spontaneous, efficient, predict-able, and easily performed. Generally, 10-15 cc of patient red blood cells can be conveniently processed in

20 this way, and the procedure can be repeated as needed.

        Example 6:        <u>Treatment of a chronic active hepatitis
                          patient with hepatitis B virus-specific T-
                          cells amplified using HLA-A2.1:GPI:hepatitis
                          B virus peptide-coated dendritic cells</u>

25       It is known that progression to chronic active hepatitis following hepatitis B virus infection is assoc-iated with the lack of T-cells reactive with certain well-defined hepatitis B virus-derived nominal antigen pep-tides. For patients with HLA-A2.1, the nominal antigen

30 peptides have been well characterized. In examples 1-4 above, the feasibility of performing protein transfer of a functional lipid-modified HLA-A2.1:hepatitis B virus nominal antigen peptide complex was illustrated. In turn, cells coated in this way can be used clinically for immu-

35 notherapeutic purposes.

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To treat a patient with chronic active hepatitis, peripheral blood is obtained from the patient by venipuncture.    Dendritic cells are cultured from the peripheral blood sample using the cytokines GM-CSF and

5     interleukin-4, as described (Romani et al., 180 J. Exp. Med. 83, 1994).    Once sufficient numbers of dendritic cells have been grown up, the cells are coated with a functional lipid-modified HLA-A2.1:hepatitis B virus nominal antigen peptide complex by simply combining the

10    two, using 10 picomolar of the recombinant protein and coincubating for two hours at 37°C in the absence of serum.    Following coating, peripheral blood mononuclear cells are added to the coated dendritic cells, and routine procedures for amplifying T-cells are followed.

15    Generally, once approximately $10^8$-$10^9$ T-cells have been accumulated via ex vivo stimulation, the cells are harvested and administered intravenously to the patient. Such T-cell transfer is now done widely and standard procedures can be followed.    In essence, the present

20    invention provides a preferable method for carrying out the T-cell amplification step.

Whereas most artificial MHC:GPI chimeric polypeptides can be loaded with nominal antigen peptide at neutral pH, some molecular combinations may require that the loading

25    step be performed at a more alkaline pH, for example, pH 5.    In the case of class II MHC, in some instances efficient loading can be obtained at neutral pH (Scheirle et al., 149 J. Immunol. 1994, 1992; Stern et al., 68 Cell 465, 1992; Sette et al., 148 J. Immunol. 844, 1992),

30    whereas in other instances, alkaline pH seems preferable (Jensen, 174 J. Exp. Med. 1111, 1991; Wettstein et al., 174 J. Exp. Med. 219, 1991).

Example 7:     A    functional    artificial    GPI-modified
               costimulator (B7-1:GPI)

35    The following example is provided to show that a B7-1:GPI molecule can function as a costimulator.    Such a

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molecule can be delivered by protein transfer methods of the claimed invention.

Recombinant GPI-modified murine B7-1 was expressed on the surface of a tumorigenic murine thymoma cell line, EL-
5  4. This was accomplished using a chimeric coding sequence in which the sequence encoding the extracellular domain of B7-1 was linked in-frame with the GPI-modification signal sequence from the 3'-end of human decay-accelerating factor. C57BL/6 mice (two per group) were injected
10  subcutaneously with either 2 X 10$^6$ mB7-1:DAF-S/BSRalphaEN-transfected (3 MB7-DS/EL4 and 4 MB7-DS/EL4) or the identical number of nontransfected (1 EL4 and 2 EL4) EL-4 tumor cells. After fourteen days, splenocytes were recovered from each animal, re-stimulated in vitro for
15  five days with irradiated nontransfected EL4 cells, and then used as effectors in a cytotoxicity assay against $^{51}$Cr-labeled EL-4 target cells at effector-to-target (E:T) ratios as shown. Splenocytes from mice immunized with B7-1:GPI-expressing EL-4 transfectants, but not from mice
20  treated with nontransfectants, demonstrated enhanced antigen-specific EL-4-directed cytotoxicity in vitro (Figure 7). Furthermore, B7-1:GPI-expressing EL-4 transfectants exhibited loss of tumorigenicity when injected into mice. This study establishes that it is
25  possible to engineer functional B7-1 derivatives with alternative modes of membrane anchorage. B7-1:GPI is a particularly interesting B7-1 derivative since it is amenable to protein transfer.

The B7-1 expression construct was produced as follows.
30  The coding sequence for the extracellular domain of murine B7-1 (Genbank Accession #X60958), from amino acids 1 to 247, was amplified by polymerase chain reaction from the murine B7-1-containing plasmid pBJ (obtained from L. Lanier). The sequences of the 5'- and 3'-primers used for
35  this PCR amplification were 5'-AAAAGCTTATGGCTTGCAATT GTGAG-3' and 5'-TTTAAGCTTGTGTTCTTGCTATCAGG-3', respectively. These primers added HindIII restriction

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endonuclease sites to both ends of the amplified B7-1
cassette.    In parallel, 3'-end DAF-S sequence was
mobilized from the plasmid pA2:DAF-S/EE6 via HindIII plus
BamHI digestion, and it was ligated into the corresponding
5     sites of the plasmid vector Bluescript (Stratagene).  The
resulting plasmid, pDAF-S/BT, was digested with HindIII
(at the upstream end of the DAF-S sequence), and the PCR-
amplified, HindIII-digested B7-1 fragment was ligated into
this site, generating the plasmid pB7-1:DAF-S/BT.    In
10    turn,    the    chimeric   B7-1:DAF-S   coding   sequence   was
mobilized using flanking XhoI and XbaI sites and was
subcloned into the corresponding sites of the eukaryotic
expression vector BSRalphaEN (obtained from D. Lublin),
generating    the    expression    construct    mB7-1:DAF-
15    S/BSRalphaEN.

     EL-4 cells, obtained from the American Type Culture
Collection,   were   transfected   with   the   mB7-1:DAF-
S/BSRalphaEN gene construct by a standard lipofection pro-
cedure according to the manufacturer's protocol (Bethesda
20    Research Laboratories).    mB7-1 epitope expression on the
surface of stable EL-4 transfectants was verified by
immunofluorescence   and   flow   cytometry.    The   staining
procedure entailed the addition of 0.25ug of CTLA4-Ig to
approximately $5 \times 10^6$ cells in a volume of 100ul for 1 hour
25    on ice.    After washing the cells three times, FITC-
conjugated goat anti-human IgG was incubated with the
cells for an additional hour.  Cells were then washed,
fixed and analyzed by flow cytometry.

     The costimulator function of B7-1:GPI was assessed
30    through a cellular immunization protocol.  According to
this protocol, C57BL/6 mice were injected subcutaneously
with    either    mB7-1:DAF-S/BSRalphaEN-transfected    or
nontransfected EL-4 tumor cells.  $2 \times 10^6$ tumor cells were
injected into each animal.  After 14 days, splenocytes
35    were recovered from each animal, washed twice, and plated
out in 24-well cell culture plates ($10^7$ cells/well) in RPMI
1640    supplemented    with    10%    fetal    bovine    serum

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(BioWittaker), 1% glutamine, 1% penicillin/streptomycin, and 10um 2-mercaptoethanol. $2 \times 10^5$ irradiated (10,000 rads) nontransfected EL-4 cells were added to each well (1:50 E:T ratio). After five days of co-incubation, cyto-
5    toxicity assays were performed using $^{51}$Cr-labeled EL-4 cells as targets. Treated splenocyte effectors were combined with the targets at different E:T ratios [$1.3 \times 10^4$ targets with either $10^6$ (E:T 76:1), $0.5 \times 10^6$ (38:1) $0.25 \times 10^6$ (19:1), or $.12 \times 10^6$ (E:T 9.6:1) effectors] in indi-
10   vidual wells of a 96-well plate and incubated for four hours at 37°C. Supernatants were harvested with Skatron harvest filters and counted in a gamma counter.

In an experiment comparing two mice in each category, there was a significant increase in CTL generation for
15   mice pre-immunized with EL-4 cells bearing GPI-modified B7-1 (Figure 7). At an effector-to-target cell ratio of 76:1, GPI-modified B7-1-primed animals showed approximately 60% specific lysis, whereas controls displayed less than 30% specific lysis. At a lower effector-to-target
20   cell ratio (9.6:1), the % specific lysis values were 45% and 25%, respectively. These data demonstrate that the B7-1:DAF-S chimeric sequence encodes a functional B7-1 polypeptide derivative.

The costimulator function of B7-1:GPI was further
25   evaluated via an assessment of its effects on EL-4 tumorigenic potential. Two syngeneic C57BL/6 mice were injected subcutaneously with $2 \times 10^6$ B7-1:GPI-expressing EL-4 transfectants, and two control animals were injected with $2 \times 10^6$ nontransfected EL-4 cells. Whereas large
30   tumors formed in all control animals by seven days post-inoculation, there was no tumor formation in the animals receiving transfected cells. This result is identical to that obtained previously with EL-4 transfectants bearing native B7-1. These results indicate that B7-1:GPI retains
35   the potential of native B7-1 to confer loss of tumorigenicity to tumor cells.

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Taken together, these data indicate that membrane anchorage via its native transmembrane hydrophobic peptide anchor is not a prerequisite for B7-1's costimulator function. This flexibility in the choice of membrane anchor opens up unique protein engineering opportunities. B7-1:GPI is of particular interest in this regard since it can be immunoaffinity purified and then reincorporated back into cell membranes.

Other embodiments are within the following claims.

## SEQUENCE LISTING

(1)  GENERAL INFORMATION:

    (i).  APPLICANT:                   Mark L. Tykocinski

    (ii)  TITLE OF INVENTION:       METHODS FOR ENGINEERING
                                      ANTIGEN-PRESENTING CELLS

    (iii)  NUMBER OF SEQUENCES:     2

    (iv)  CORRESPONDENCE ADDRESS:

        (A)    ADDRESSEE:        Lyon & Lyon
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                              Suite 4700
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        (E)    COUNTRY:        U.S.A.
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    (v)  COMPUTER READABLE FORM:

        (A)    MEDIUM TYPE:      3.5" Diskette, 1.44 Mb
                              storage
        (B)    COMPUTER:        IBM Compatible
        (C)    OPERATING SYSTEM: IBM P.C. DOS 5.0
        (D)    SOFTWARE:        Word Perfect 5.1

    (vi)  CURRENT APPLICATION DATA:

        (A)    APPLICATION NUMBER:  08/324,125
        (B)    FILING DATE:      October 14, 1994
        (C)    CLASSIFICATION:   Not Yet Classified

    (vii)  PRIOR APPLICATION DATA:

        Prior applications total,
        including application
        described below:        1

        (A)    APPLICATION NUMBER:  08/260,547
        (B)    FILING DATE:      June 16, 1994

    (viii)    ATTORNEY/AGENT INFORMATION:

        (A)    NAME:                     Warburg, Richard J.
        (B)    REGISTRATION NUMBER:    32,327
        (C)    REFERENCE/DOCKET NUMBER:  209/244

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        (A)    TELEPHONE:              (213) 489-1600
        (B)    TELEFAX:                (213) 955-0440
        (C)    TELEX:                   67-3510


(2)    INFORMATION FOR SEQ ID NO:    1:

      (i)    SEQUENCE CHARACTERISTICS:

        (A)    LENGTH:            26 base pairs
        (B)    TYPE:               nucleic acid
        (C)    STRANDEDNESS:     single
        (D)    TOPOLOGY:         linear

      (ii)    SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AAAAGCTTAT GGCTTGCAAT TGTGAG                        26


(2)    INFORMATION FOR SEQ ID NO:    2:

      (i)    SEQUENCE CHARACTERISTICS:

        (A)    LENGTH:            26 base pairs
        (B)    TYPE:               nucleic acid
        (C)    STRANDEDNESS:     single
        (D)    TOPOLOGY:         linear

      (ii)    SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTTAAGCTTG TGTTCTTGCT ATCAGG                        26

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## Claims

1.  A method for producing a cell having a defined MHC:nominal antigen peptide on its membrane comprising the step of:

5       a)      contacting the external portion of said cell with an externally applied nominal antigen peptide and an artificial lipid-modified MHC polypeptide able to bind said nominal antigen peptide.

2.  A method for producing a cell having a defined
10  MHC:nominal antigen peptide on its membrane comprising the steps of:

        a)      first contacting the external portion of said cell with an externally applied artificial lipid-modified MHC polypeptide able to bind a nominal antigen
15  peptide; and

        b)      second contacting said cell with said nominal antigen peptide so that said artificial lipid-modified MHC polypeptide binds said peptide.

3.  A method for producing a cell having a defined
20  MHC:nominal antigen peptide on its membrane comprising the steps of:

        a)      first contacting an nominal antigen peptide and an artificial lipid-modified MHC polypeptide able to bind a nominal antigen peptide; and

25      b)      second contacting the external portion of said cell with externally applied nominal antigen peptide bound to said artificial lipid-modified MHC polypeptide.

4.  A method for producing a biological membrane having a defined MHC:nominal antigen peptide on its
30  surface comprising the step of:

        a)      contacting said membrane with a nominal antigen peptide and an artificial lipid-modified MHC polypeptide able to bind said nominal antigen peptide.

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5.     A  method  for  producing  a  biological  membrane
having  a  defined  MHC:nominal  antigen  peptide  on  its
surface comprising the steps of:

a)    first  contacting  said  membrane  with  an
artificial  lipid-modified  MHC  polypeptide  able  to  bind  a
nominal antigen peptide; and

b)    second  contacting  said  membrane  with  said
nominal  antigen  peptide  so  that  said  artificial  lipid-
modified  MHC  polypeptide  binds  said  peptide.


6.     A  method  for  producing  a  biological  membrane
having  a  defined  MHC:nominal  antigen  peptide  on  its
surface comprising the steps of:

a)    first  contacting  a  nominal  antigen  peptide
and  an  artificial  lipid-modified  MHC  polypeptide  able  to
bind  a  nominal  antigen  peptide;  and

b)    second  contacting  said  membrane  with  said
nominal  antigen  peptide  bound  to  said  artificial  lipid-
modified  MHC  polypeptide.


7.     The  method  of  claim  1,  2,  3,  4,  5,  or  6  wherein
said  MHC  polypeptide  comprises  a  class  I  MHC  polypeptide
sequence.


8.     The  method  of  claim  1,  2,  3,  4,  5,  or  6  wherein
said  MHC  polypeptide  comprises  a  class  II  MHC  polypeptide
sequence.


9.     The  method  of  claim  1,  2,  3,  4,  5,  or  6  wherein
said  lipid-modified  MHC  polypeptide  comprises  a  glycosyl-
phosphatidylinositol-modified  MHC  polypeptide.


10.  The  method  of  claim  9  wherein  said  GPI-modified
MHC  polypeptide  is  produced  by  the  steps  of:

a)    gene  transfer  of  a  chimeric  gene  expression
construct  comprising  a  GPI  modification  signal  sequence
into  a  host  cell;and

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b) isolation of said GPI-modified MHC polypeptide from said host cell.

11. A method for activating an antigen-specific T-cell comprising the step of contacting a cell having a
5  defined MHC:nominal antigen peptide complex on its membrane with a T-cell population comprising a T-cell with specificity for said MHC:nominal antigen peptide complex.

12. A method for inhibiting an antigen-specific T-cell comprising the step of contacting cell having a
10 defined MHC:nominal antigen peptide complex on its membrane with a T-cell population comprising a T-cell with specificity for said MHC:nominal antigen peptide complex.

13. The method of claim 11 or 12 wherein said contacting step is performed ex vivo.

15     14. The method of claim 11 or 12 wherein said contacting step is performed in vivo.

15. The method of claim 11 or 12 wherein said MHC polypeptide is syngeneic with said antigen-specific T-cell.

20     16. The method of claim 11 or 12 wherein said MHC polypeptide is allogeneic with said antigen-specific T-cell.

17. The method of claim 11 wherein said modified cell is a dendritic cell.

25     18. The method of claim 11 wherein said modified cell is an activated B-cell.

19. The method of claim 12 wherein said modified cell is a cell lacking a costimulator.

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20.    The method of claim 11 wherein said modified cell is presenting a tumor antigen.

21.    The method of claim 12 wherein said modified cell is expressing a coinhibitor.

5       22.    The method of claim 1, 2, 3, 4, 5, or 6 wherein said lipid-modified MHC polypeptide comprises a polyhistidine tag.

23.    A glycosyl-phosphatidylinositol-modified MHC polypeptide which is separate from a cell membrane.

10      24.    A cell having a membrane exogenously coated with a lipid-modified MHC polypeptide.

25.    A modified antigen-presenting cell comprising a cell with an artificial costimulator polypeptide exogenously reincorporated into the surface of said cell.

15      26.    The modified antigen-presenting cell of claim 25 comprising an immunogenic tumor cell.

27.    The modified antigen-presenting cell of claim 25 wherein said artificial costimulator polypeptide comprises a glycosyl-phosphatidylinositol moiety.

20      28.    The modified antigen-presenting cell of claim 25 wherein said artificial costimulator polypeptide comprises a streptavidin moiety.

29.    The modified antigen-presenting cell of claim 25 wherein said artificial costimulator polypeptide comprises
25  a molecule with affinity for a molecule on the surface of said cell.

30. The modified antigen-presenting cell of claim 25 wherein said cell is pre-coated with a molecule with affinity for an artificial costimulator polypeptide.

31. The modified antigen-presenting cell of claim 25
5 wherein the costimulator portion of said artificial costimulator polypeptide is selected from the group consisting of B7-1, B7-2, B7-3, intracellular adhesion molecule-1, intracellular adhesion molecule-2, intracellular adhesion molecule-3, LFA-3, VCAM-1, and
10 fibronectin.

32. A method for producing a modified antigen presenting cell having a costimulator on its membrane comprising the step of:
        a)    contacting the external portion of said cell
15 with an externally applied artificial costimulator polypeptide.

33. The method of claim 32 wherein said costimulator polypeptide comprises an artificial lipid-modified costimulator polypeptide.

20    34. The method of claim 33 wherein said artificial lipid-modified costimulator polypeptide comprises a glycosyl-phosphatidylinositol moiety.

35. The method of claim 32 wherein said costimulator polypeptide is selected from the group consisting of B7-1,
25 B7-2, B7-3, intracellular adhesion molecule-1, intracellular adhesion molecule-2, intracellular adhesion molecule-3, LFA-3, VCAM-1, and fibronectin.

36. A method for enhancing a cell's immunogenicity comprising contacting said cell with a membrane-binding
30 artificial costimulator polypeptide.

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37. A method for inducing anti-tumor immunity against an autologous tumor cell in a patient comprising the step of:

5      a)    increasing the amount of a costimulator molecule on the surface of said tumor cell by protein transfer.

38. A method for inducing anti-tumor immunity against an autologous tumor cell in a patient comprising the step of:

10     a)    administering to said patient an autologous tumor cell with externally reincorporated costimulator polypeptide on its membrane.

39. A method for inducing anti-tumor immunity against an autologous tumor cell in a patient comprising the step

15 of:

       a)    administering    to   said    patient    an heterologous·tumor cell with externally reincorporated costimulator polypeptide on its membrane.

40. A method for inducing anti-tumor immunity against

20 an autologous tumor cell in a patient comprising the step of:

       a)    administering to said patient a tumor cell membrane    extract    with    externally    reincorporated costimulator polypeptide.

25     41. A method for identifying a molecule that enhances the    immunogenicity   of   an   antigen   presenting   cell, comprising the step of:

       a)    coating said cell with a membrane-binding polypeptide derivative of said molecule, and

30     b)    determining   the   immunogenicity   of   the resulting cell compared to an uncoated cell.

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42. The method of claim 41 wherein said antigen-presenting cell is a tumor cell.

43. The method of claim 42 wherein determining the immunogenicity comprises determining the capacity of said
5 coated tumor cell to induce an anti-tumor immune response.

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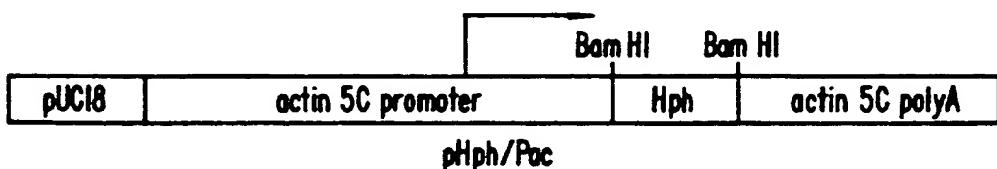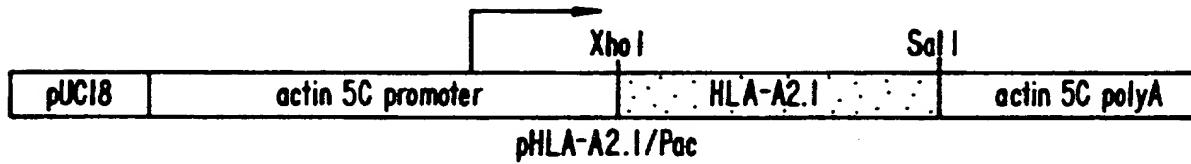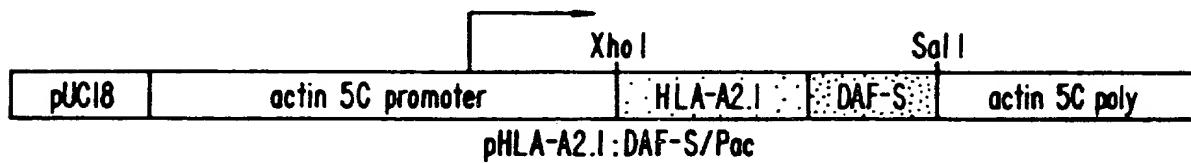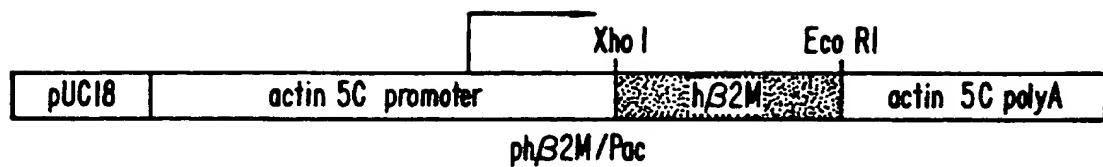


FIG. 1.

FIG. 2.

FIG. 3.

LOG FLUORESCENCE INTENSITY

—— 1 pmole

--- 0.2 pmoles

···· 0.1 pmoles

—— 0 pmoles

NUMBER OF CELLS

B

FIG. 4b.

LOG FLUORESCENCE INTENSITY

—— 60 MINUTES

···· 30 MINUTES

— — 10 MINUTES

—— 1 MINUTES

—— 0 MINUTES

NUMBER OF CELLS

A

FIG. 4a.

FIG. 4d.

LOG FLUORESCENCE INTENSITY

NUMBER OF CELLS

D

—— CIR
—— CIR+HLA-A2.I:GPI
---- CIR+HLA-A2.I:GPI+MA58-66
—— CIR+HLA-A2.I:GPI+HBV env 335-343

FIG. 4c.

LOG FLUORESCENCE INTENSITY

NUMBER OF CELLS

C

—— CIR+HLA-A2.I:GPI, 4°C
····· CIR+HLA-A2.I:GPI,37°C
---- CIR+HLA-A2.I:GPI+PI-PLC,37°C
—— CIR+PI-PLC
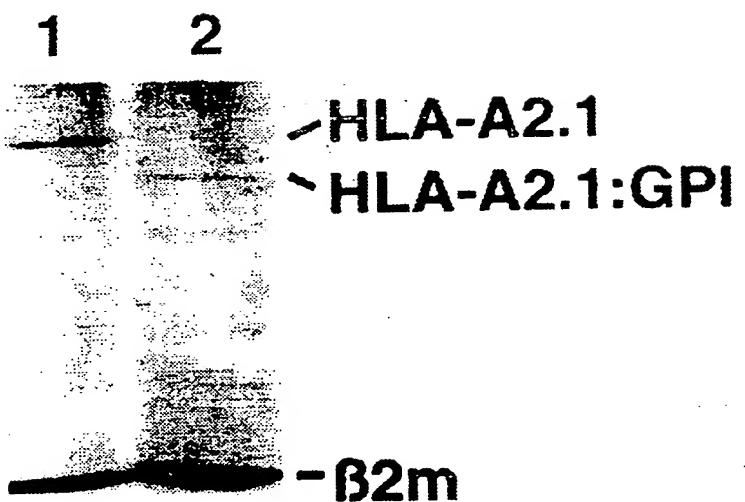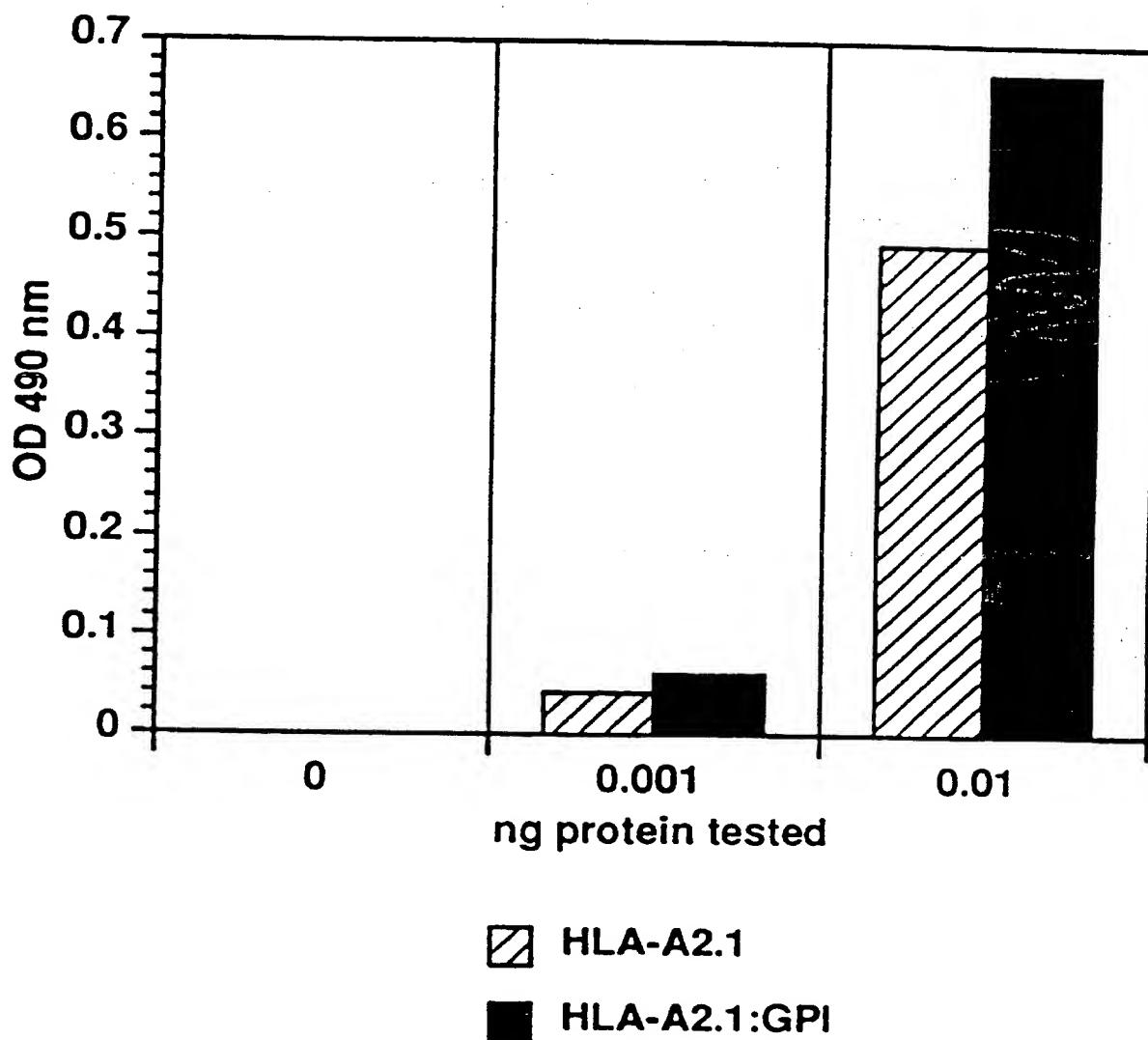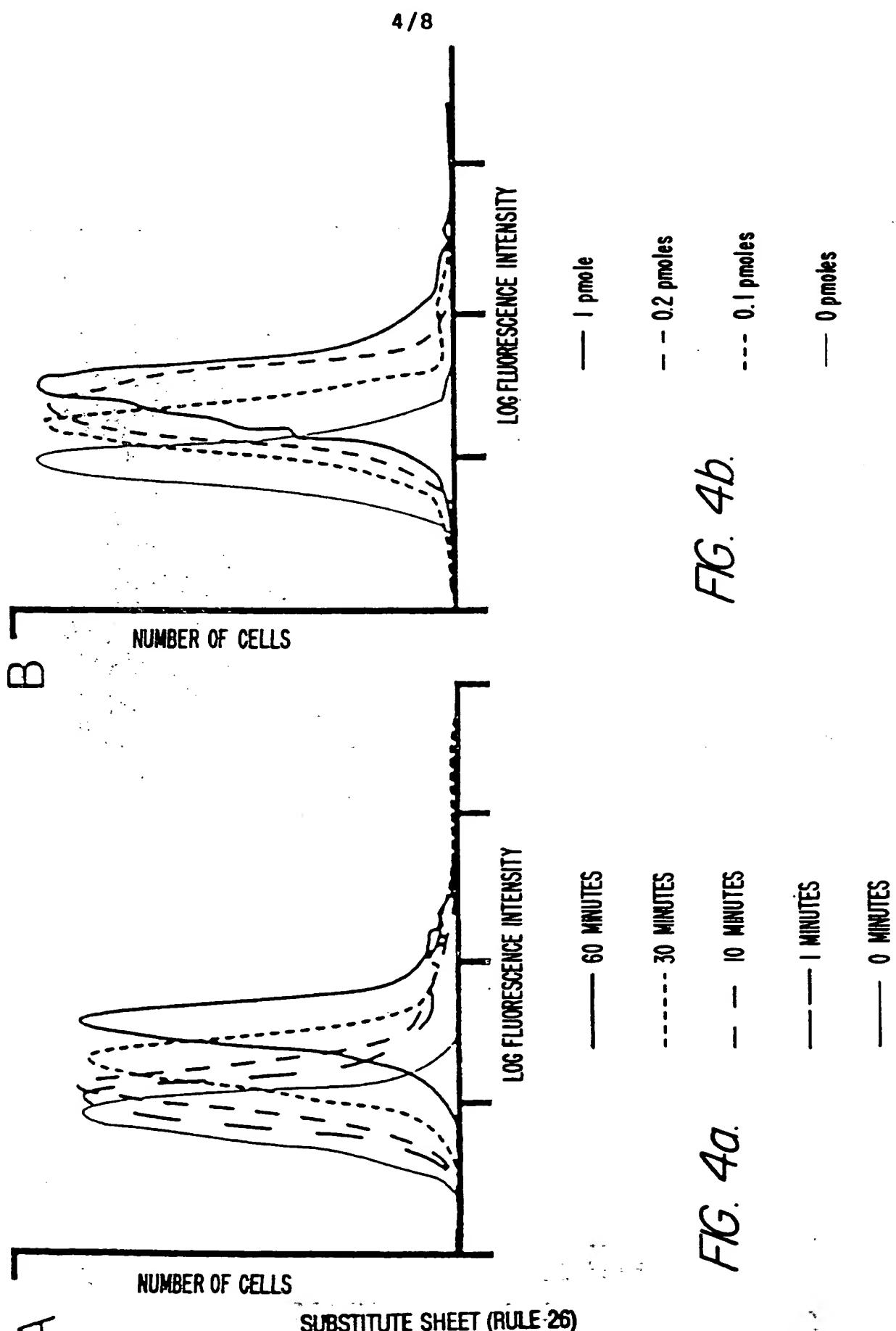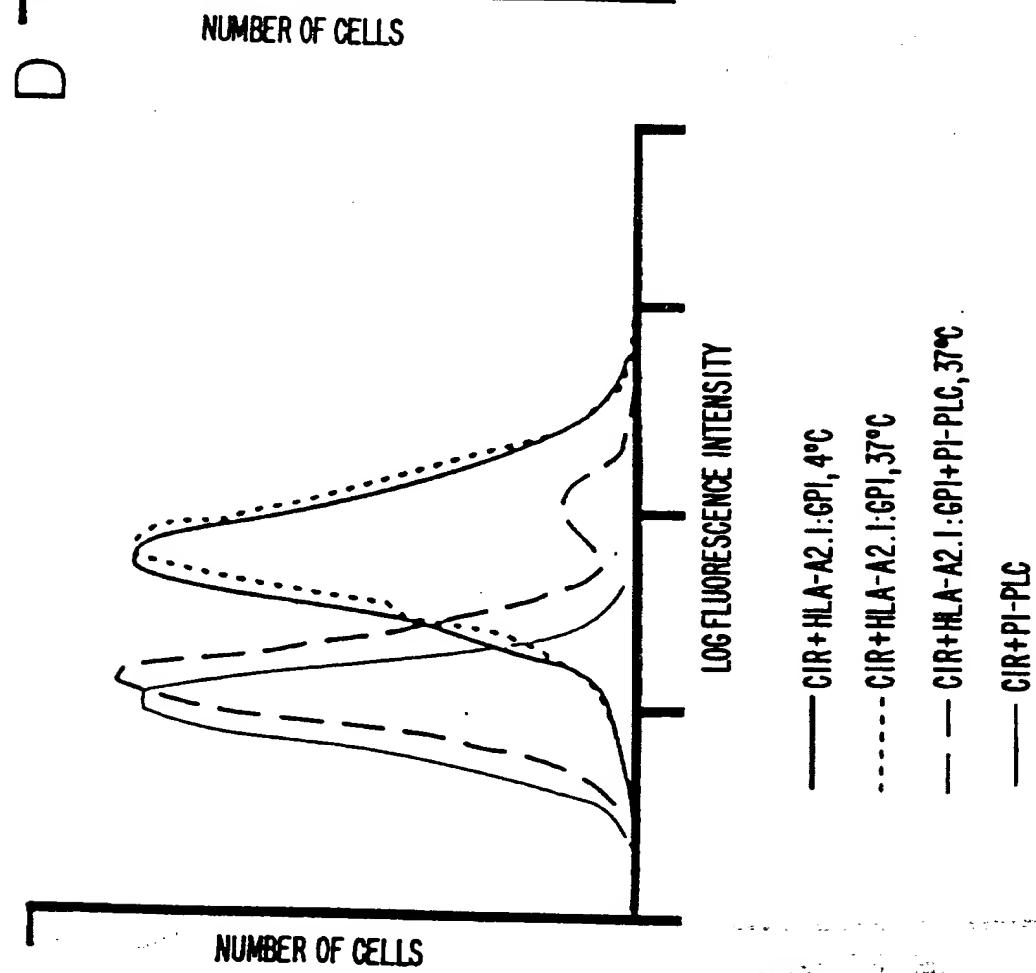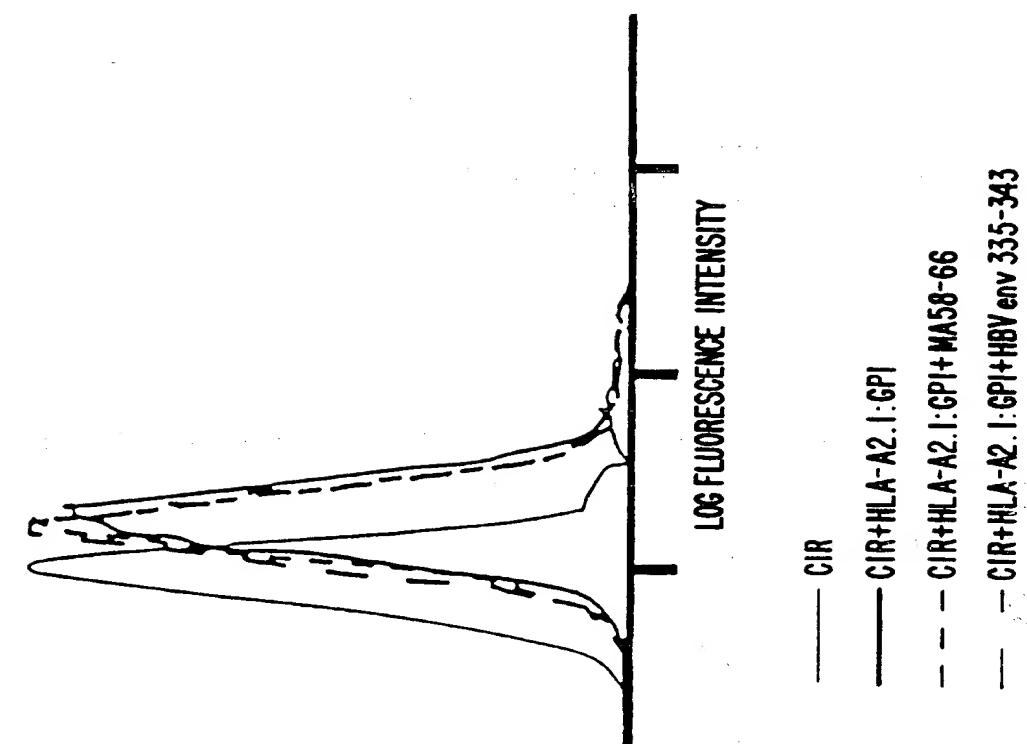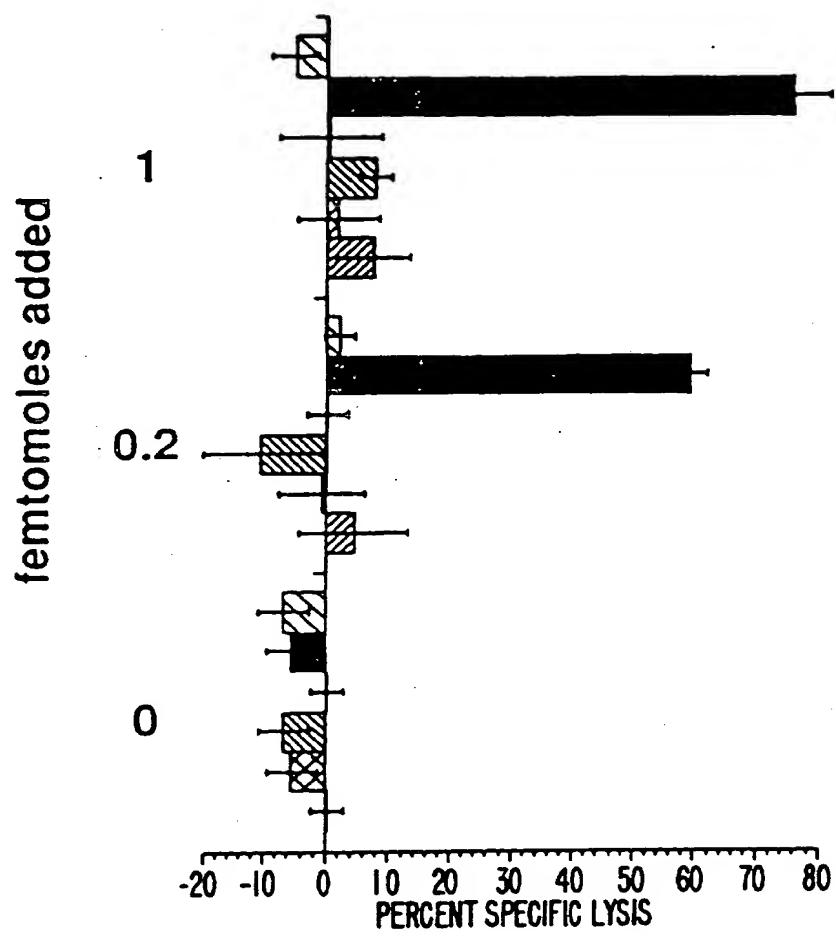
◻ HLA-A2.1:GPI + MA58-66

◼ HLA-A2.1:GPI + HBV env335-343

▨ HLA-A2.1:GPI

▨ HLA-A2.1 + MA58-66

⊠ HLA-A2.1 + HBV env335-343

▨ HLA-A2.1

## FIG. 5.

FIG. 6.

FIG. 7.

FIG. 1.

*FIG. 2.*

*FIG. 3.*

FIG. 4b.

FIG. 4a.

D

NUMBER OF CELLS

LOG FLUORESCENCE INTENSITY

——— C1R

——— C1R+HLA-A2.1:GPI

– – – C1R+HLA-A2.1:GPI+NA58-66

——— C1R+HLA-A2.1:GPI+HBV env 335-343

FIG. 4d.



C

NUMBER OF CELLS

LOG FLUORESCENCE INTENSITY

——— C1R+HLA-A2.1:GPI,4°C

········· C1R+HLA-A2.1:GPI,37°C

– – – C1R+HLA-A2.1:GPI+PI-PLC,37°C

——— C1R+PI-PLC

FIG. 4c.

FIG. 5.

FIG. 6.

FIG. 7

# PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

**(54) Title:** METHODS FOR ENGINEERING ANTIGEN-PRESENTING CELLS

**(57) Abstract**

A protein transfer method for producing a cell having a defined MHC: nominal antigen peptide or costimulator on its membrane.

phβ2M/Pac

pHLA-A2.1:DAF-S/Pac

pHLA-A2.1/Pac

pHph/Pac

# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | IMMUNITY,<br>vol. 1, 1994,<br>pages 607-613, XP002009544<br>HUANG, J.-H. ET AL.: "Protein transfer of preformed MHC-peptide complexes sensitizes target cells to T cell cytolysis"<br>*see the whole article* | 1-43 |
| X | JOURNAL OF CELLULAR BIOCHEMISTRY,<br>vol. Suppl. 16D, 1992,<br>pages 64-abstract O 424, XP002009545<br>HUANG, J.-H. ET AL.: "Expression and functional analysis of a recombinant human GPI anchored class I human leukocyte" | 23,24 |
| Y | *see the whole article* | 1-43 |

-/--

[X] Further documents are listed in the continuation of box C.     [ ] Patent family members are listed in annex.

*Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 29 July 1996 | 2 0. 08. 96 |

| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Authorized officer<br><br>Marie, A |

# INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | MOLECULAR IMMUNOLOGY, vol. 31, no. 13, 1994, pages 1017-1028, XP002009546 HUANG, J.-H. ET AL: "Alloantigenic recognition of artificial GPI-anchored HLA-A2.1" | 23,24 |
| Y | *see the whole article* | 1-43 |
| X | JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 3, no. 4, 1992, pages 895-906, XP002009547 D. BROWN ET AL.: "GPI-anchored membrane proteins" | 23,24 |
| Y | *see the whole article | 1-43 |
| X | JORNAL OF IMMUNOLGY, vol. 152, no. 11, 1994, pages 5268-5274, XP002009548 P. TABACZEWSKI ET AL.: "Expression of secreted and GPI-bound Qa-2 molecules is dependent on functional TAP-2 peptide transporter" | 23,24 |
| Y | *see the whole article* | 1-43 |
| X | INTERNATIONAL IMMUNOLGY, vol. 4, no. 6, 1992, pages 673-680, XP002009549 R. GREENLAW ET AL.: "Transfection of HLA-DR-expressing DAP.3 cells with a cDNA clone encoding the GPI-linked form of LAF-3: biochemical features and functional consequences" | 25-27 |
| Y | *see the whole article* | 1-43 |
| X | JOURNAL OF EXPERIMENTAL MEDICINE, vol. 178, 1993, pages 1893-1901, XP002009550 P. PAGLIA ET AL.: "Immortalized dendritic cell line fully competent in antigen presentation initiates primary T cell responses in vivo" | 25-27 |
| Y | *see the whole text* | 1-43 |

-/--

1

International Application No

**PCT/US 95/12718**

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | JOURNAL OF IMMUNOLOGY, vol. 149, no. 5, 1992, pages 1556-1561, XP002009551 S. D. NORTON ET AL. : "The CD28 lignad, B7, enhances IL-2 production by providing costimulatory signal to T cells" | 25-27 |
| Y | *see the whole article* | 1-43 |

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